The Establishment Of Two Novel Bovine Cell Lines By Transfection With The Putative Transforming Region Of Bovine Adenovirus Type 3

by

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ABSTRACT

Human adenoviruses (Ads), members of the family *adenoviridae*, are medium-sized DNA viruses which have been used as valuable research tools for the study of RNA processing, oncogenic transformation, and for the development of viral vectors for use in gene delivery and immunization technology. The left 12% of the linear Ad genome codes for products which are necessary for the efficient replication of the virus, as well as being responsible for the formation of tumors in animal models. The establishment of the *293* cell line, by immortalization of human embryonic kidney cells with the E1 region of Ad type 5 (Ad5), has facilitated extensive manipulation of the Ads and the development of recombinant Ad vectors.

The study of bovine adenoviruses (BAVs), which cause mild respiratory and gastrointestinal infections in cattle has, on the other hand, been limited primarily to that of infectivity, immunology and clinical manifestations. As a result, any potential as gene delivery vehicles has not yet been realized. Continued research into the molecular biology of BAVs and the development of recombinant vectors would benefit from the development of a cell line analogous to that of the *293* cells.

In an attempt to establish such a cell line, the recombinant plasmid pKC-*neo* was constructed, containing the left 0-19.7% of the BAV type 3 (BAV3) genome, and the selectable marker for resistance to the aminoglycoside G418, a neomycin derivative. The plasmid construct was then used to transfect both the Madin-Darby bovine kidney (MDBK)

cell line and primary bovine lung cells, after which G418-resistant foci were selected for analysis. Two cell lines, E61 (MDBK) and E24 (primary lung), were subsequently selected and analysed for DNA content, revealing the presence of the pKC-*neo* sequences in their respective genomes. In addition, BAV3 RNA transcripts were detected in the E61 cells. Although the presence of E1 products has yet to be confirmed in both cell lines, the E24 cells exhibit a phenotype characteristic of partial transformation by E1. The apparent immortalization of the primary lung cells will permit exploitation of their ability to take up exogenous DNA at high efficiency.

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This place has made all the difference. Thanks.

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ABBREVIATIONS

Ad	adenovirus
ATF-2	activating transcription factor two
BAV	bovine adenovirus
bp	base-pair
cAMP	cyclic adenosine monophosphate
cdk	cyclin dependent protein kinase
CFTR	cystic fibrosis transmembrane regulatory region
CMV	cytomegalovirus
CR	conserved region
CRE	cAMP responsive element
CTL	cytotoxic T lymphocyte
dATP	deoxyadenosine
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
ds	double-stranded
E	adenovirus early region
E2F factor	E2 transcription
E4F factor	E4 transcription
ITR	inverted terminal repeat
kDa	kilo-dalton
L	adenovirus late region
LT	SV40 large tumor antigen
mCi	millicurrie
MDBK	Madin-Darby bovine kidney
MHC	major histocompatibility complex
MLP	major late promoter
mRNA	messenger RNA
mT	SV40 middle tumor antigen
mu	map unit
NFIII	nuclear factor three
ORF	open reading frame
RB	retinoblastoma
ß-gal	ß-galactosidase
SV40	Simian virus type 40
TBP	TATA-binding protein
TFIID	transcription factor two D
TNF	tumor necrosis factor
	triphosphate
	triphosphate
wt	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

For my dogs, Baby and Abe.

Thanks, guys.

CHAPTER 1: INTRODUCTION

Adenoviruses are medium-sized, non-enveloped DNA viruses, which cause mild respiratory and gastrointestinal disorders in a variety of hosts (Straus, 1984). The adenovirus genome consists of a single molecule of double-stranded (ds) DNA, having an average length of 36,000 base-pairs (bp), depending on the particular serotype, a classification based on several criteria, such as the agglutination of rat erythrocytes (Hierholzer, 1973). Six early (E) and five late (L) transcriptional units are distributed over both strands of the genome, the E regions coding primarily for regulatory proteins, and the L regions coding for structural proteins of the viral capsid. The E and L designations refer to the time of expression, either before or following replication of the viral genome.

The left 12% of the human adenovirus (Ad) genome codes for products which are responsible for the efficient transcription of the other viral regions, as well as for oncogenic transformation in experimental systems. This region is designated E1, being the first expressed during infection. The products of E1 exert their effects through association with cellular transcription factors, in a mechanism of *trans*-regulation, making this region a valuable tool for studying the regulation of transcription in eukaryotic cells.

The *trans*-regulatory nature of the E1 products has been exploited to facilitate the replication of Ad mutants having deletions in their E1 sequences, which are otherwise unable to replicate in most mammalian cells. Rescue of these mutants has been accomplished by supplying the E1 products from the expression of E1 sequences which have been inserted into the genome of the host cell. In 1986, Haj-Ahmad and Graham reported the first of such experiments, in the construction of an Ad type 5 (Ad5) mutant having deletions in E1 and another early region, E3 (Haj-Ahmad and Graham, 1986). The Ad5 mutant of Haj-Ahmad and Graham was rescued in the *293* cell line, which contains and expresses the E1 region of Ad5, resulting from the immortalization of human embryonic kidney cells with this same region (Graham *et. al.*, 1977).

Bovine adenoviruses (BAVs) are a subgroup of adenoviruses which infect bovine and ovine species, but have not been studied to nearly the same extent as the human Ads. As a result, any potential for clinical applications has not yet been realized. The development of a system for the manipulation and study of BAVs and BAV vectors, analogous to the human Ad system, necessitates the establishment of a cell line analogous to that of *293* cells, but expressing the E1 products of a BAV.

The development of a recombinant BAV vector has appeal, not only for use in veterinary medicine, but for the potential in human gene therapy and immunization, eliminating the pitfalls of undesirable reversion, through recombination, to a wild-type (wt) state, which might result in symptomatic infections and the

inappropriate delivery of genes to non-target tissues, a concern in the case of the human Ad vectors.

The construction of a cell line analogous to *293* cells is described here, based on the introduction of BAV type 3 (BAV3) E1 sequences into both the Madin-Darby Bovine Kidney (MDBK) cell line, an established cell line, as well as into primary bovine lung cells. The strategy behind the construction of the novel cell line consisted of three main elements. The first goal was the insertion of the BAV3 sequences into the established MDBK cell line. The second goal involved an attempt to immortalize primary cells using the putative transforming properties of the BAV3 E1 region, properties which are predicted based on results with the analogous E1 region of the human Ads. The third goal, pending success of the second, was the establishment of a new cell line having properties desirable for BAV research in general. Since there have been relatively few detailed studies of the biological properties of BAVs, BAV research could benefit from such a cell line.

CHAPTER 2: LITERATURE REVIEW

2.1 The adenovirus genome

The adenovirus, a medium-sized DNA virus, is a member of the family *adenoviridae*, which is subdivided into aviadenoviruses and mastadenoviruses (figure 1). Animal adenoviruses are responsible for mild infections of the upper respiratory and gastrointestinal tracts of humans and other animals (Straus, 1984), with the human subgroup (Ads) first being isolated from adenoidal tissue of children suffering from upper respiratory infections (Rowe *et. al.*, 1953). The bovine adenoviruses (BAVs) are a subgroup of the animal adenoviruses, and although much of the following discussion concerns the extensively studied human subgroup, the basic features of the virus and its genome are shared by the BAVs, which will be discussed in more detail in a later section.

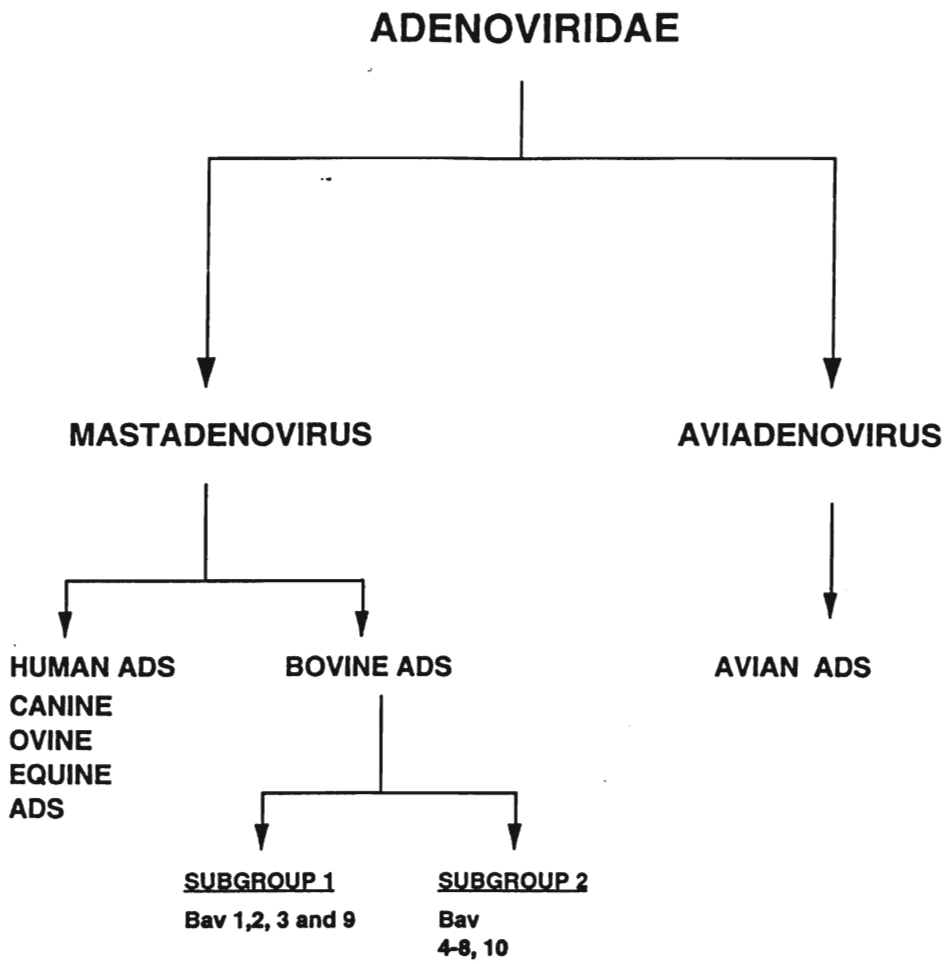


Figure 1. Members of the family *Adenoviridae*.

The Ads contain a linear, double-stranded molecule of DNA, having an average length of 36,000 bp, depending on the particular serotype. Conventional maps of the Ad genome are divided into 100 increments, or map units (mu), each unit corresponding from approximately 325 to 360 bp in length, depending on the serotype (figure 2). The two strands of the genome are labelled, by convention, right (r) and left (l), pertaining to the direction of transcription from the numerous coding regions. These regions are differentiated by the relative times at which they are transcribed during infection. Four early (E) regions, coding for regulatory proteins, are transcribed from six promoters, located on both the r and the l strands. A single late promoter, the major late promoter (MLP), drives transcription of all five late (L) regions, which code for the structural proteins of the viral capsid, on the r strand. The coding regions are flanked by inverted terminal repeats (ITRs) of 100 to 200 bp, which contain the origins of replication and consensus sequences recognized by nuclear factors, such as NFIII.

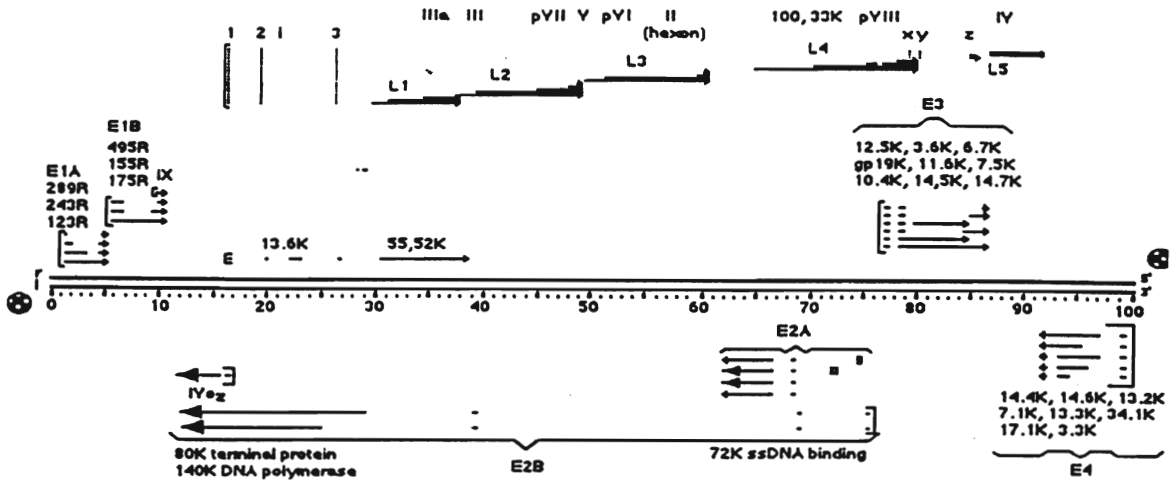


Figure 2. Conventional map of the Ad2 genome. One map unit corresponds to approximately 360 bp. The mRNA and protein products are listed above and below the map, relative to the locations on the genome from which they are expressed. (arrows) mRNA; ([]) promoters; (E,L) transcriptional units (from Joklik, 1988).

2.2 Early region 1

The first region to be expressed during infection, E1, is required for the efficient transcription of the other early genes and thus required for initiation of viral replication (Nevins, 1981; Berk, 1986). E1 of Ad2 and Ad5 is divided into two transcription units, E1A (1.3-4.5 mu) and E1B (4.6-11.2 mu) (Grand, 1987; Nevins, 1987). Both E1A and E1B code for several mRNA and protein products, where transcripts are labelled according to their sedimentation coefficient (S). The mRNA transcription products of E1A share common 5' and 3' ends, and are generated by alternative splicing of the primary RNA transcripts. Similarly, the E1B transcripts share 3' sequences, and are also generated through alternative splicing, as well as the use of different reading frames.

The E1A codes for 5 mRNA products, the 13S, 12S, 11S, 10S and 9S transcripts (figure 3). The E1A mRNA can be detected as early as 30 minutes follwing infection (Berk *et. al.*, 1979), but only the 12S and 13S products accumulate at this stage. The 9S mRNA appears at late stages of infection (Berk and Sharp, 1978), and, along with the 10S and 11S transcripts, codes for products which have not been well characterized. In Ad2 and Ad5, the 12S and 13S transcripts code for products of 243 and 289 amino acids, respectively, designated 243R and 289R (Perricaudet *et. al.*, 1979; Ricciardi *et. al.*, 1981; Gaynor *et. al.*, 1982). These proteins are identical except for a stretch of 46 amino acids, present in the 289R product, from residue 140 to residue 188, but absent in the 243R product (Moran and Mathews, 1987). This unique 46 amino acid sequence constitutes one of three

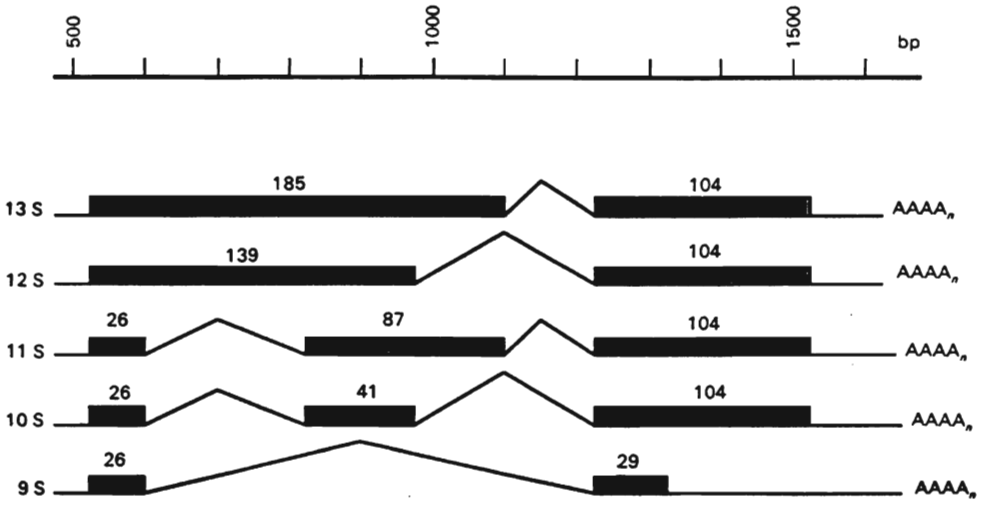


Figure 3. Splicing events and gene products from the E1A region of Ad2. The solid line at the top of the figure represents the Ad2 genome from nucleotide 500 (1.3mu) to nucleotide 1600 (4.5mu). The RNA transcripts are shown as solid lines, labelled according to the sedimentation coefficient (S) of the mRNA products. Proteins are shown as boxes, with the number corresponding to the number of amino acid residues. Splicing events are shown as angled lines. (from Boulanger and Blair, 1991).

regions which are highly conserved among most adenovirus serotypes, designated CR (conserved region) 1, CR2 and CR3, which are responsible for various effects, described in figure 4 (Lillie *et. al*, 1987). The E1A products are diffusible *trans*-activators (Berk, 1986; Jones *et. al.*, 1988; Nevins, 1987), and it is the unique region, CR3, which is responsible for this function, and therefore for the expression of all other adenoviral genes (Moran and Mathews, 1987).

Genetic analysis, using deletions and point mutations in the E1A region, has shown that the 243R and 289R E1A proteins stimulate transcription from all of the early promoters, as well as from the E1A promoter itself. The transcription from the E1A promoter, however, is stimulated by only 5 fold, as compared to the other promoters, which are stimulated 50 fold by the E1A proteins (Berk *et. al.*, 1979; Jones and Shenk, 1979; Osborne *et. al.*, 1984). The E1A promoter is also under partial control of the E1B region, but it is not clear if this is due to *trans*-regulation or to a *cis* effect (Barker and Berk, 1987; Grand, 1987; Senear and Lewis, 1986), although expression of E1A is greatly enhanced in transfection experiments when encoded on the same plasmid as E1B, as opposed to coinfection with a separate plasmid carrying E1B (Jochemsen *et. al.*, 1987).

The E1A products stimulate transcription from class II and class III promoters, which are transcribed by RNA polymerase II and RNA polymerase III, respectively (Leong and Berk, 1986; Berger and Folk, 1985; Berk, 1986) Transactivation by the 289R E1A product can be attributed to interactions with the TATA-binding protein (TBP), a component of the transcription initiation complex TFIID,

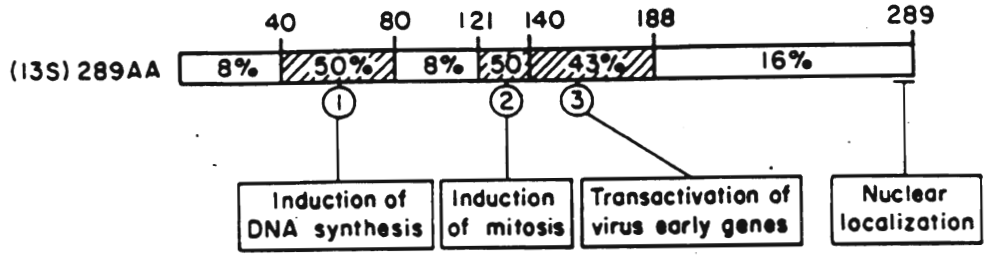


Figure 4. Regulatory regions of the 289R product of E1A. Conserved regions and degree of homology between several Ad serotypes are shown in hatched boxes. Amino acid positions are indicated above the bar, and the function of each region below the bar. (from Moran and Mathews, 1987).

demonstrated by retention of TBP on E1A affinity columns (Horikoshi *et. al.*, 1991). In addition, transcription from E1B, which is dependent on E1A expression, is diminished if the TATA box within the upstream regulatory regions is interrupted (Wu *et. al.*, 1987). The CR3 appears to contain the TBP-binding motif, since deletions or mutations in this region inhibits formation of the TBP-E1A complex (Lee *et. al.*, 1991). Some mutations, however, do not disturb the complex, but affect transactivation, suggesting that other proteins are required for the complete transactivation events. There are suggestions that E1A might in fact form a bridge between ATF-2 (a DNA-binding transcription factor) and TBP (Lee *et. al.*, 1991).

E1A has been found to act synergistically with cyclic AMP (cAMP), a second-messenger of signal-transduction pathways, which regulates gene expression by a cascade of events involving protein kinases (Engel *et. al.*, 1988). cAMP responsive elements (CRE) have been located in upstream regulatory sequences of cellular and viral genes, and transcriptional activation of E1A, E1B and E4 occurs in the presence of cAMP (Engel *et. al.*, 1988). In addition, activating transcription factor (ATF) binds to and activates the CRE in the promoters of E1A, E3 and E2 (Hurst and Jones, 1987; Leza and Hearing, 1989; Lin and Green, 1988). The synergistic effect of E1A and cAMP induce the DNA-binding activity of another cellular transcription factor, AP-1 (Muller *et. al.*, 1989), which recognizes the CRE and AFT consensus sequences (Angel *et. al.*, 1987), suggesting a role in transactivation of viral genes. Although E1A and cAMP act synergistically, both resulting in greater induction of transcription

than with either inducer alone (Engel *et. al.*, 1988), they act by different pathways (Leza and Hearing, 1989).

The cellular transcription factors E2F and E4F, discovered by their ability to bind to elements of the E2 and E4 promoters, are also stimulated by the products of E1A (Reichel *et. al.*, 1988; Raychaudhuri *et. al.*, 1987). This activation, which results in a dramatic increase in the DNA-binding activity of both factors (Reichel *et. al.*, 1987), may be, however, an indirect consequence of the presence of E1A products, and is more directly associated with a product of another early region, E4 (Neill *et. al.*, 1990).

Transcription from the E1B region produces 5 mRNA species, the 22S, 13S, 14S, 14.5S and 9S transcripts (figure 5). The overlapping 22S, 13S, 14S and 14.5S products are transcribed from a common promoter, which is activated by the E1A products (Dery *et. al.*, 1987), and are generated by alternative splicing of a common RNA precursor (Pettersson *et. al.*, 1983). The 9S mRNA is transcribed from an independent promoter, and is expressed 6-8 hours after infection, coding for a structural component of the viral capsid (Boulanger *et. al.*, 1979).

Two protein products, of 19kDa (p19) and 55kDa (p55), are produced from the 22S E1B transcript, using AUG codons from different reading frames (Bos *et. al*, 1981). The other mRNAs, except for the 9S transcript, also code for the same p19 product, resulting in the accumulation of this protein during infection (Boulanger and Blair, 1991). The role of p19 in the infectious cycle seems to be the maintenance of the integrity of viral and cellular DNA (Stillman, 1986), and thereby indirectly increasing trancription from newly

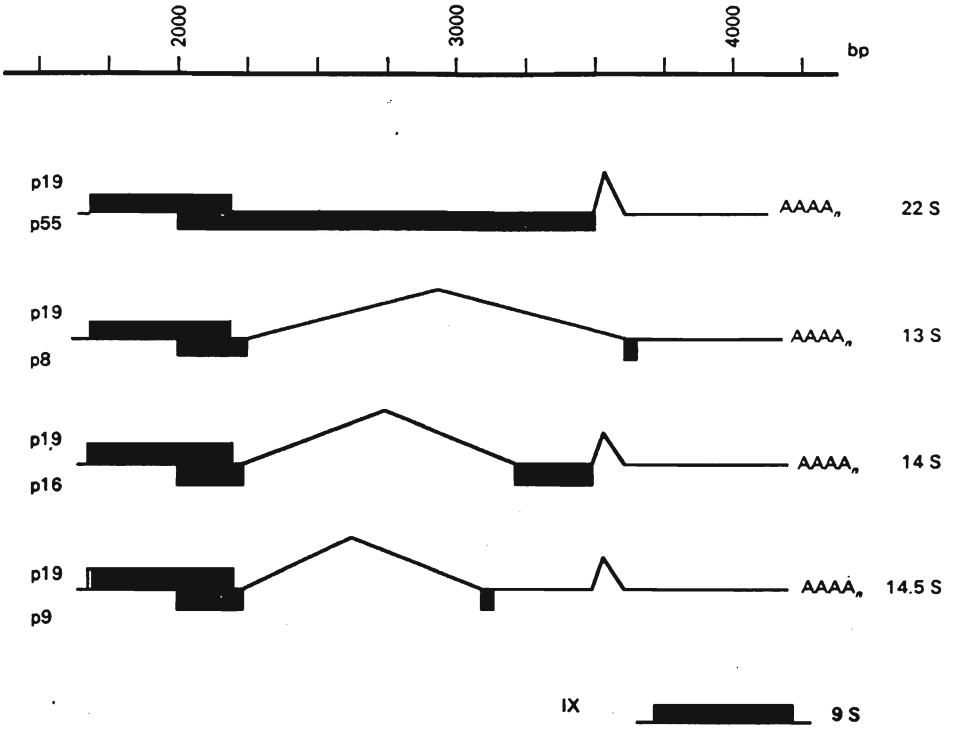


Figure 5. Splicing events and gene products from the E1B region of Ad2. The solid line at the top of the figure represents the Ad2 genome from 4.6 mu to 11.2 mu. The RNA transcripts are shown as solid lines, labelled according to the sedimentation coefficient (S) of the mRNA products. Proteins are shown as boxes, and are labelled according to their molecular weight (kDa), from p9 (9kDa) to p55 (kDa). Splicing events are shown as angled lines. (from Boulanger and Blair, 1991).

introduced viral DNA (Herrmann and Mathews, 1989), perhaps by inhibition of a cellular nuclease activity at the low pH characteristic of Ad infections (D'Halluin *et. al.*, 1985). The E1B p19 also stimulates expression from early Ad promoters, including E1A (Herrman *et. al.*, 1987; Senear and Lewis, 1986).

The p55 protein also has a necessary role for the virus life-cycle. Mutations in p55 have been associated with defective growth and delayed replication of viral DNA (Stillman, 1986). The activity of p55 appears to be required for shut-off of cellular DNA synthesis, as well as for efficient viral DNA replication and the maturation of mRNAs expressed from the late regions. This latter suggestion comes from mutants of p55 which are defective for the production of structural capsid proteins. The defect appears to lie at a point between the completion of transcription and transport of the mRNA across the nuclear membrane (Babiss *et. al.*, 1985; Barker and Berk, 1987). This p55 function seems to result from a complex with the p34 protein product of E4, since mutations in the p34 open-reading frame (ORF) results in low levels of viral mRNA in the cytoplasm and reduced shut-off of cellular protein synthesis, effects observed in the presence of p55 mutations (Halbert *et. al.*, 1985).

The interaction of the E1 products with cellular factors involved in the regulation of the cell cycle has implications which go beyond the regulation of viral replication. In particular circumstances, E1 products can induce changes in cellular pathways and, in turn, alter the phenotype of the cell. This property has placed the Ads into a special group of viruses, those responsible for the formation of some tumors.

2.3 Oncogenic transformation and E1

Ads are members of the DNA tumor viruses, a group which also includes the polyomaviruses, hepadnaviruses, papillomaviruses, herpesviruses and poxviruses. These viruses are capable of conferring phenotypic changes in mammalian cells, giving them characteristics of tumor cells, and have been found to be associated with tumor formation in whole animals.

Cells which have been phenotypically altered in such a way are said to have been *transformed*. Transformed cells have characteristic growth properties and morphological features which distinguish them from their normal counterparts. The oncogenic transformation of mammalian cells is a two-stage process (Land *et. al.*, 1983). The first stage is the establishment of *immortalization*, resulting in cells which are capable of indefinite growth in culture, presumably due to the deletion or mutation of one or more senescence genes, or a mechanism which overrides the action of the scenescence gene(s) (Pereira-Smith and Smith, 1988). Such cells are commonly used as research tools, since their growth is not limited by the natural constraints imposed on primary cell cultures, which have a finite life-span of 20-100 generations (Freshney, 1994). All of these cell lines have resulted from either spontaneous or induced immortalization of normal, healthy cells.

The second stage of oncogenic transformation involves the aquisition of growth characteristics and morphological features which are not associated with normal cells, nor are they typical of immortalized cells. These properties include a reduction in growth

serum requirements, the loss of contact inhibition, decreased anchorage dependence of growth, as well as changes on the cell surface, in the cytoskeleton, and in the extracellular matrix (Bowen-Pope *et. al.*, 1984; Freshney, 1994; Topp *et. al.*, 1981). Transformed cells may exhibit any combination of these properties, but do not necessarily display all of them (Topp *et. al.*, 1981).

In all cases of transformation, whether induced by such diverse sources as a virus, a chemical carcinogen or radiation, the fundamental change is of a genetic nature. Genes which underly the events of oncogenesis, *oncogenes*, may be introduced into the cell as foreign DNA or, if already present in the cell, may be the target of mutation.

The introduction of oncogenes is the most common mechanism of transformation by tumor viruses. In the case of RNA viruses, specifically retroviruses, the oncogene is an altered copy of a normal cellular counterpart, or *proto*-oncogene. This phenomenon raises questions concerning the origin of these oncogenes, since their existence in such diverse genomes as those of animals and viruses implies that one had aquired a copy from the other, with the transmission from animal to virus being the favoured explanation, since these genes have no apparent role in the retroviral genome.

The mutated form of the cellular proto-oncogene carried in a retrovirus disrupts the normal events of cell-cycle control when introduced into the target genome. The discovery of the retrovirus oncogenes has, as a result, led to the identification of the cellular homologues, and the delineation of the respective functions in the cell. Some of these oncogenes are listed in table 1, along with the

function of the cellular proto-oncogenes, the source of the virus and the associated tumors.

The genes delivered by DNA tumor viruses, on the other hand, do not have homologues normally shared by the cell, but are genuine viral genes. Among these viruses, the Ads have been extensively studied in terms of their oncogenes, particulary since the discovery of the oncogenic potential of Ad12 in 1962 (Trentin *et. al.*, 1962), a discovery which propelled Ads into the forefront of molecular biological research, with the discovery of RNA splicing being among the resulting major advancements (Berget *et. al.*, 1977; Chow *et. al.*, 1977). One of the observations emerging from Ad-mediated oncogenic research was that DNA virus oncogenes exert their effect through interference with normal cellular pathways, as opposed to retroviral oncogenes, which are integral parts of those pathways.

Although tumors associated with Ad infections have never been observed in nature, Ads are capable of transforming cells which do not support productive infections, as was the case in the initial 1962 discovery, whereby Ad12 was responsible for tumor formation in newborn hamsters (Trentin *et. al.*, 1962). Such non-permissive cells do not facilitate productive lytic cycles for particular Ad groups, as a result of a block to the late stages of infection (Doerfler, 1991). Consequently, non-permissive or abortive infection may result in the

Table 1. Sources and functions of retroviral oncogenes, and their associated tumors (from Alberts *et. al.*, 1994).

Oncogene	Proto-oncogene Function	Source of Virus	Virus-induced Tumor
abl	protein kinase (tyrosine)	mouse cat	pre-B-cell leukemia sarcoma
erb-B	protein kinase (tyrosine): epidermal growth factor (EGF) receptor	chicken	erythroleukemia, fibrosarcoma
fes	protein kinase (tyrosine)	cat/chicken	sarcoma
fms	protein kinase (tyrosine): macrophage colony-stimulating factor (M-CSF) receptor	cat	sarcoma
fos *jun*	products associate to form AP-1 gene regulatory protein	mouse chicken	osteosarcoma fibrosarcoma
kit	protein kinase (tyrosine): Steel factor receptor	cat	sarcoma
raf	protein kinase (serine/threonine) activated by Ras	chicken/ mouse	sarcoma
myc	gene regulatory protein of the HLH family	chicken	sarcoma; myelocytoma, carcinoma
H-*ras*	GTP-binding protein	rat	sarcoma; erythroleukemia
K-*ras*	GTP-binding protein	rat	sarcoma; erythroleukemia
rel	gene regulatory protein related to NFκB	turkey	reticuloendotheliosis
sis	platelet-derived growth factor, B chain	monkey	sarcoma
src	protein kinase (tyrosine)	chicken	sarcoma

capable of immortalizing cells in culture, the lack of oncogenicity in whole animals might be due to a mechanism independent of the immortalizing and transforming functions. In Ad12-transformed cells there is a down-regulation of the products of the major histocompatibility complex (MHC) (Eager *et. al.*, 1985; Schreier *et. al.*, 1983; Vasarada *et. al.*, 1986), which might enable these cells to escape cytotoxic T lymphocyte (CTL) surveillance, and result in their tumorigenicity (Tanaka *et. al.*, 1985; Bernards *et. al.*, 1983). This event might be mediated by a 19kDa product of another early region, E3, which was shown to complex with MHC class 1 in the endoplasmic reticulum (Kvist *et. al.*, 1978), as well as a 14.7kDa product from the same region, which prevents lysis by tumor necrosis factor (TNF) (Gooding *et. al.*, 1988). Differential degrees of sequestration by these E3 products between Ad12 and Ads2 and 5 may explain the differences in tumorigenicity.

Another explanation for the different degrees of tumorigenicity among Ad groups may be differences in viral functions involved in inserting the viral DNA into the host cell, in such a way that it can be efficiently expressed (Rowe and Graham, 1983). This explanation suggests that the degree of oncogenicity is independent of the source of the E1 region, once it is being expressed.

Expression of both E1A and E1B is required for complete transformation of primary cells. The products of E1A are alone capable of immortalizing primary cells in culture, being both necessary and sufficient for this event (Houweling *et. al.*, 1980) and, in the presence of other oncogenes, such as *ras* or the E1B products, can result in transformation (Branton *et. al.*, 1985; Ruley, 1983; Zerler

integration of viral DNA sequences into the host-cell genome. This integration event, as in the case of Ad12 infection of the rodent cells, may result in oncogenic transformation.

Cells which have been transformed by this mechanism contain, as a minimum, the left 0-12% of the Ad genome stably integrated into their own genome, and constituitively express the products of the E1A and E1B regions (Graham and van der Eb, 1973; Gallimore *et. al.*, 1985). This observation, consistent in all cases of transformation by Ads, implicated the E1 region as that responsible for the transformation event. The ability of the E1 region alone, as the only input DNA, to transform cells which are otherwise permissive to Ad infection is consistent with the notion that integration of the Ad genome, as would be the fate in the case of an abortive or non-permissive infection, is the requirement for transformation (Graham *et. al.*, 1977).

The products of E1 are recognized by serum antibodies from animals bearing tumors of adenoviral origin (Matsuo *et. al.*, 1982). As a result, they are referred to as tumor antigens, or T-antigens, a term which is also used to describe similar products from other tumor viruses, such as the large-T (LT) antigen of Simian virus 40 (SV40).

The various serotypes of the human Ads are not equally oncogenic, and are classified based on their ability to form tumors in newborn rodents (Graham, 1984). In contrast to the highly oncogenic Ad12, Ad2 and Ad5 are classified as non-oncogenic *in vivo*. Since the E1 region of both Ad2 and Ad5 are nonetheless

capable of immortalizing cells in culture, the lack of oncogenicity in whole animals might be due to a mechanism independent of the immortalizing and transforming functions. In Ad12-transformed cells there is a down-regulation of the products of the major histocompatibility complex (MHC) (Eager *et. al.*, 1985; Schreier *et. al.*, 1983; Vasarada *et. al.*, 1986), which might enable these cells to escape cytotoxic T lymphocyte (CTL) surveillance, and result in their tumorigenicity (Tanaka *et. al.*, 1985; Bernards *et. al.*, 1983). This event might be mediated by a 19kDa product of another early region, E3, which was shown to complex with MHC class 1 in the endoplasmic reticulum (Kvist *et. al.*, 1978), as well as a 14.7kDa product from the same region, which prevents lysis by tumor necrosis factor (TNF) (Gooding *et. al.*, 1988). Differential degrees of sequestration by these E3 products between Ad12 and Ads2 and 5 may explain the differences in tumorigenicity.

Another explanation for the different degrees of tumorigenicity among Ad groups may be differences in viral functions involved in inserting the viral DNA into the host cell, in such a way that it can be efficiently expressed (Rowe and Graham, 1983). This explanation suggests that the degree of oncogenicity is independent of the source of the E1 region, once it is being expressed.

Expression of both E1A and E1B is required for complete transformation of primary cells. The products of E1A are alone capable of immortalizing primary cells in culture, being both necessary and sufficient for this event (Houweling *et. al.*, 1980) and, in the presence of other oncogenes, such as *ras* or the E1B products, can result in transformation (Branton *et. al.*, 1985; Ruley, 1983; Zerler

et. al., 1986). The specific contributions of these regions to immortalization and transformation are shown in figure 6, and a summary of their overall participation in viral replication and cellular transformation is presented in figure 7.

The transforming properties of the E1 region have been mapped by deletion analysis to CR1 and CR2 of the 289R and 243R products, between amino acids 40 to 80, and 121 to 139 (Kuppuswamy and Chinnadurai, 1987; Lillie *et. al.*, 1987; Moran and Zerler, 1988). These regions contain sites for interactions with a member of a group of cellular proteins known as tumor-suppressors, or anti-oncogenes. The deletion or inactivation of the tumor-suppressor genes and their products are associated with many human tumors (Lee *et. al.*, 1988). The association of E1A products with tumor-suppressors has therefore been implicated as the mechanism behind the oncogenic activities of E1A.

One of the predominant tumor-suppressor targets of the E1A proteins is the product of the retinoblastoma-susceptibility gene (*RB*). Cells which lack functional *RB* products undergo tranformation, underlying the development of familial *retinoblastoma*. This anti-oncogenic product, p105-*RB*, has been found to be physically associated with the E1A proteins *in vitro* and *in vivo* (Egan *et. al*, 1989; Whyte *et. al.*, 1988). One of the cellular targets of p105-*RB* is the cellular transcription factor E2F, which controls the transcription of several cellular proliferation genes (Nevins, 1992). Association of p105-*RB* with E2F regulates progression through G1, by exerting negative control on E2F (Schwartz *et. al.*, 1993). This p105-*RB*:E2F complex is disrupted by the E1A products *in vitro*, by direct

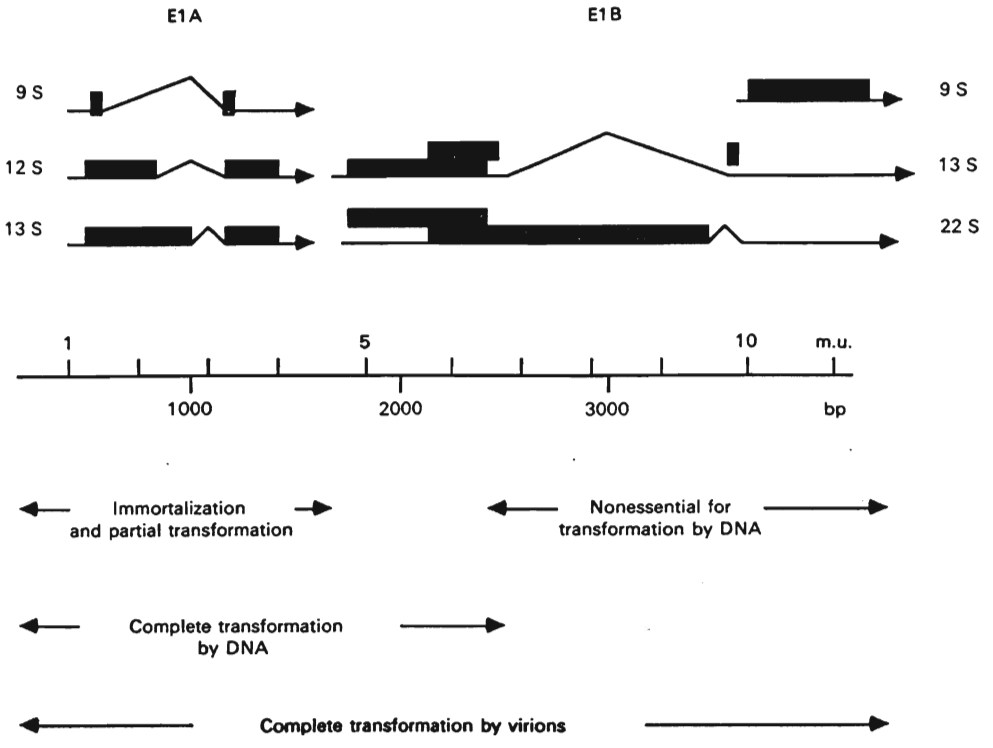


Figure 6. Structure and functions of the oncogenic regions E1A and E1B of Ad2. The solid line in the middle of the figure represents the Ad2 genome from 0-12 mu. The top half of the figure shows the RNA (solid lines) and protein (boxes) products of the E1A and E1B regions. The lower half of the figure describes the contribution of the particular regions in immortalization and transformation. (from Graham, 1984).

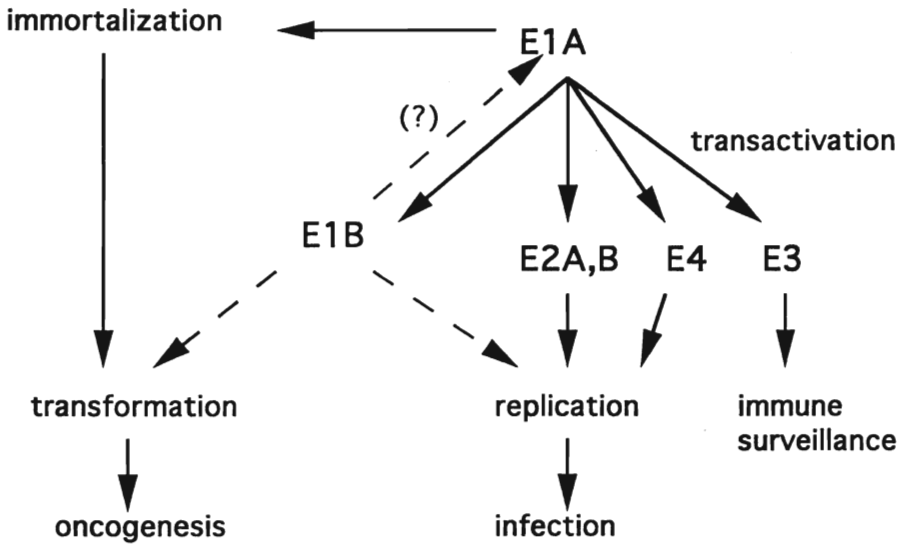


Figure 7. Summary of E1A and E1B participation in viral replication and cellular transformation. (E) early region.

interaction between p105-*RB* and the CR1 and 2 domains of the E1A proteins (Bandara and LaThangue, 1991; Whyte *et. al.*, 1988). This dissociation event is believed to result in the release of cell cycle control by liberation of the E2F transcription factor. The net result, however, seems to be the prevention of entry into G_0 in quiescent cells, rather than the direct, positive induction of proliferation, since the disruption of the p105-*RB*:E2F complex by E1A occurs too slowly to account for the rapid entry into S-phase observed in E1A-transformed cells (Spitkovsky *et. al.*, 1994; Wang *et. al.*, 1991).

The rapid induction of proliferation by E1A therefore seems to be the result of an additional mechanism, one which seems to involve the cyclins, which induce proliferation through activation of the cyclin-dependent protein kinase (*cdk*) gene family products (Pines, 1993). Regulation of cell-cycle progression by the cyclins is demonstrated by overexpression of any of the cyclin genes A, D1 or E, which can overcome the block to cell division imposed by pRB (Hinds *et. al.*, 1992), and the observation that overexpression of cyclins E and D1 can accelerate the G1 phase in fibroblasts (Quelle *et. al.*, 1993). Spitkovsky *et. al.* (1994) demonstrated that expression of both cyclins A and E are induced by E1A, showing that E1A-dependent S-phase entry is correlated to modulation of cyclin gene expression, and that the disruption of the p105-*RB*:E2F complex may not be required for the onset of DNA synthesis in quiescent rat fibroblasts. The control exerted by E1A through the cyclins reveals the ability of E1A to mimick serum-dependent cell-cycle activation.

The requirement for both E1A and E1B expression for complete transformation may be attributed to both the p19 and p55 products of E1B. Clues into the role of p19 in cellular transformation arise from the cellular localization of this product. The p19k protein has been found in such diverse locations as the nuclear envelope and the endoplasmic reticulum (White *et. al.*, 1984; Blair-Zajdel *et. al.*, 1985), as well as associated with the plasma membrane (Persson *et. al.*, 1982; Smith *et. al.*, 1989). In addition, this protein has also been found to be associated with the vimentin-containing intermediate filament network (White and Cipriani, 1989, 1990). It is this last observation that implicates p19 in contributing to the transformed phenotype, in particular, the anchorage-independent growth characteristic of the transformed cells, by disruption of the intermediate filament network. In the case of tumor formation in whole animals, the E1B p19 product protects the infected cell against the cytotoxic effects of tumor-necrosis factor (TNF), reminiscent of the role of E3, allowing oncogenesis to proceed (White *et. al*, 1992).

The role of E1B p55 in transformation may be analogous to that of E1A, since the protein can form a complex with the tumor-suppressor product p53, resulting in a release of growth control (Finlay *et. al.*, 1989; Lane and Benchimol, 1990). In addition, there have been suggestions that p55 may function at the level of DNA integration in a transformed cell (Rowe and Graham, 1983).

The necessity for the role of p55 in transformaton by whole virus was demonstrated by Babiss *et. al.* (1984) and Graham *et. al.* (1977), followed by a demonstration of the requirement for expression of both p19 and p55 in DNA-mediated transformation,

which was confirmed by genetic analysis using point-mutations (Senear and Lewis, 1986; Barker and Berk, 1987). The cooperative nature of E1A and E1B is a general phenomenon of oncogenes, and is not unique to the adenovirus. Oncogenes can be classified into 2 broad groups. The first group, which includes E1A, are only able to immortalize primary cells in culture, locking them in a proliferative mode (Land *et. al.*, 1983). This group also includes products such as the SV40 LT antigen and those of *myc*, proteins which are generally localized in the nucleus. Structural studies have, in fact, revealed a similarity between the products of E1A and those of *myc* and *myb* (McLachlan and Boswell, 1985; Ralston and Bishop, 1983). The second group consists of cytoplasmic or membrane-bound proteins, such as the *ras* products, polyomavirus mT antigen and the membrane-associated p19 E1B protein. These proteins are responsible for completion of the transformation events (Land *et. al.*, 1983; Byrd *et. al.*, 1988; Kelekar and Cole, 1987; Ruley, 1983). The complementary nature of transformation by these elements is particulary apparent in the ability of the polyomavirus mT antigen and the products of *ras* to substitute for E1B in transformation by E1A (Ruley, 1983).

2.4 Adenovirus vectors

The mechanism of transformation by the adenovirus has been a topic of extensive research, contributing to a detailed understanding of some of the adenovirus products. In addition, the adenovirus, having a relatively simple genome, has proved to be an invaluable research tool to study the events which control gene

expression in mammalian cells. As a result, an understanding of the structural and functional aspects of the adenovirus genome eventually, and perhaps inevitably, led to the manipulation of the adenovirus for exploitation as a vector for the delivery and expression of foreign genes in mammalian cells.

Adenovirus vectors are currently being used in pre-clinical trials of gene therapy for the treatment of cystic fibrosis (Crystal *et. al.*, 1994). The adenovirus is an attractive vector for such an ailment, because of its tropism for tissues of the respiratory and the gastrointestinal tract, which, in the case of cystic fibrosis, facilitates delivery to the patient by aerosol. The adenovirus is advantageous as a vector because of its ability to express genes in non-dividing cells, not only in cells such as those just mentioned, but also in the liver (Jaffe *et. al.*, 1992), blood vessels (Lemarchand *et. al.*, 1993), muscle (Stratford-Perricaudet *et. al.*, 1992) and brain (Le Galle La Salle *et. al.*, 1993). This advantage is in contrast to retrovirus vectors, which are quite effective, but only for rapidly dividing cells such as stem cells which, incidentally, must be removed from the patient and treated in culture, followed by reintroduction to the bone marrow. As with the case in the cystic fibrosis trials, the adenovirus can be delivered to the whole animal, intravenously or otherwise.

The possibility of rescuing a virus from mammalian cells, using an input merely of plasmid DNA, was realized in 1983 by Kathleen Berkner and Philip Sharp (Berkner and Sharp, 1983). When plasmids containing overlapping fragments of the Ad5 genome, together encompassing the entire genome, were cotransfected into a human embryonic cell line (the *293* cell line), the result was the

production of wt virus. The results of the experiment opened the door for the construction and rescue of adenovirus mutants, whereby regions of the genome could be manipulated in a convenient plasmid form, followed by reconstruction *in vivo*.

The work of Berkner and Sharp was complemented by Haj-Ahmad and Graham, who used this method of homologous recombination and viral rescue to construct the first adenovirus mutant used to carry and express foreign genes in mammalian cells. Indeed, the adenovirus vector developed by Haj-Ahmad and Graham has been used extensively since their initial report in 1986 (Haj-Ahmad and Graham, 1986), to express exogenous genes in numerous animal and cellular models, and is currently being used in clinical trials for the delivery of the cystic fibrosis transmembrane regulatory (CFTR) protein to the respiratory tract of individuals suffering from cystic fibrosis, as previously mentioned (Crystal *et. al.*, 1994).

The double-deletion mutant of Haj-Ahmad and Graham, *dl*E1,3, was constructed to accomodate the packaging of additional DNA into the viral genome, since wt Ad can package only up to 105% of its genome (Ghosh-Choudhury *et. al.*, 1986), or an additional 2 kilobases (kb) of DNA. The mutant contained deletions in both E1 and E3, eliminating 5.5 kbp of the genome, and making room for an additional 7.5 kbp of exogenous DNA (Haj-Ahmad and Graham, 1986). The construction of *dl*E1,3, using the method of intracellular recombination, is illustrated in figure 8.

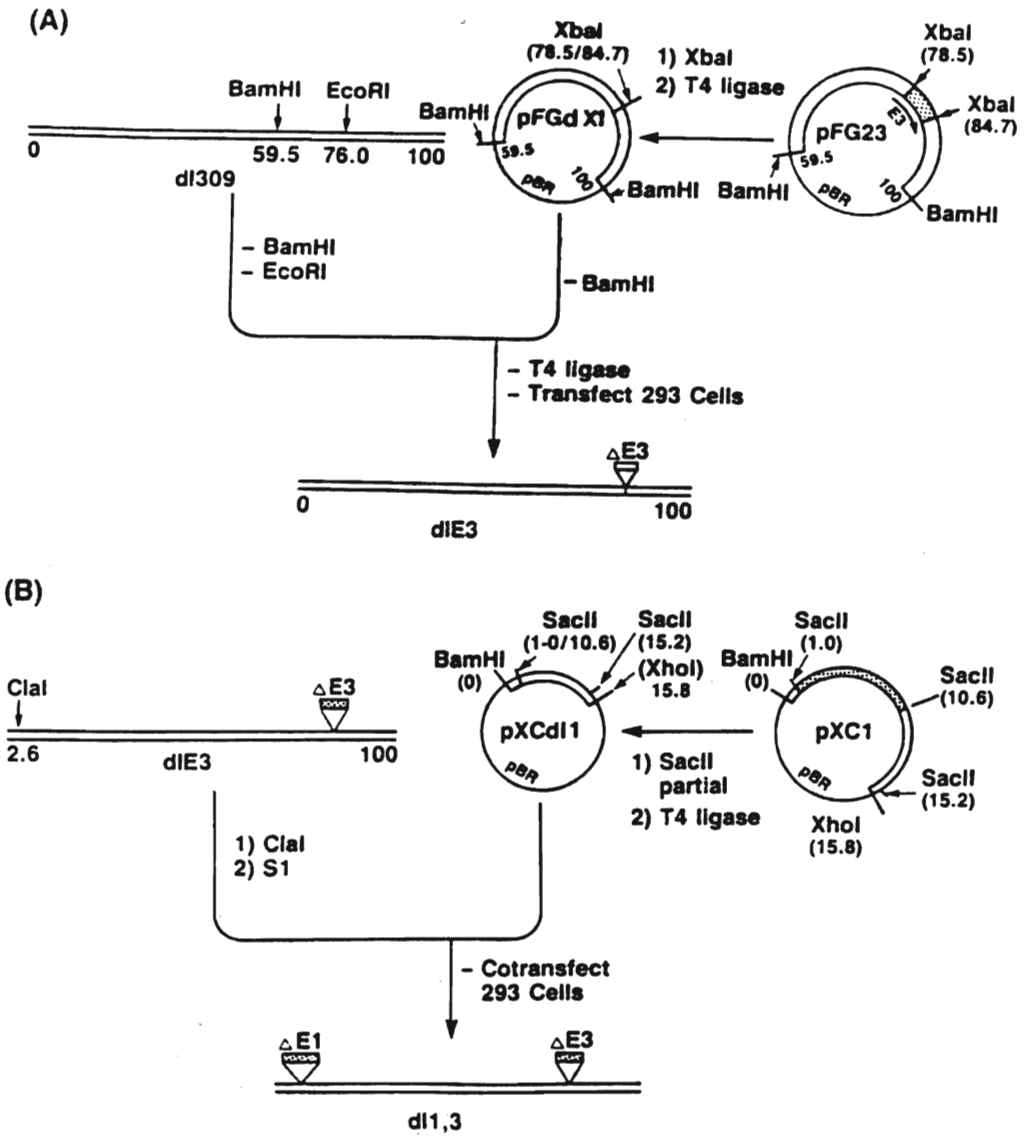


Figure 8. Construction of the double-deletion Ad5 mutant, *dl*E1,3. (from Haj-Ahmad and Graham, 1986). (A) Deletion of E3 from 78.5 to 84.7 mu, generating *dl*E3. (B) Deletion of E1 from 1 to 9.2 mu. *dl*E1,3 was rescued by recombination between *dl*E3 and the E1 deletion, in *293* cells (B).

The choice of regions E1 and E3 for the location of the deletions was based on 1) observations that region E3 is non-essential for viral growth and replication (involved in downregulation of the immune response), and 2) the availability of a cell line (*293* cells), which constituitively expressed the products of the E1 region (Graham *et. al.*, 1977). Adenoviruses which lack the E1 region are normally unable to replicate in a host cell, but by supplying the missing functions *in trans*, the *293* cells can accomodate growth of the deletion-mutants, facilitating their culture.

The removal of the essential E1 region was the means by which the desired control over the adenovirus infective cycle was accomplished. The double-deletion mutants were ideally suited for applications in gene therapy, incapable of lytic infection in the target tissues.

The deletion of E1 was placed so as to maximize the packaging capacity, while maintaining the integrity of other essential regions. The left-most border of the deletion in E1 is defined by adenoviral sequences from 0 to 1 mu, which must be present on the viral genome (ie. *in cis*) for replication, and which, therefore, cannot be supplied by the *293* cells. These sequences include the left ITR, the origin of replication, and a signal for encapsidation of complete virions. The right-most border is limited by the coding region for a capsid protein (protein IX), which is required for packaging of full-length genomes (Ghosh-Choudhury *et. al.*, 1986).

2.5 Bovine adenoviruses

The discussion to this point has been concerned only with the human adenoviruses. There are, however, *adenviridae* groups which infect animal hosts other than humans, as well as those which infect birds. These non-human adenoviruses have not been studied to anywhere near the extent of the human subgroups and, as a result, have not been exploited as viral vectors. The genus *mastadenovirus* contains the human Ads, as well as subgroups which infect monkeys, cattle, pigs, sheep, horses, dogs, goats and mice (Joklik, 1988). The potential for exploration and manipulation of non-human adenoviruses is, therefore, large enough to warrant further research.

Of the animal adenoviruses, the bovine adenoviruses (BAVs) have been characterized to an extent sufficient to place them in a position of consideration for manipulation. In particular, important regions of the BAV type 3 (BAV3) genome, including the putative E1A and E1B, as well as E3 of BAV2, have been sequenced and characterized by the laboratory of Haj-Ahmad (Elgadi *et. al.*, 1993; Esford and Haj-Ahmad, 1994). BAV3 is a member of the group I BAVs, of which serotypes 1, 2 and 9 are the other members. A second group of BAVs (group II) consists of serotypes 4-8 (Bartha, 1969).

Particular aspects of the E1 sequence of BAV3 suggests that, for the most part, reading frames of the human Ads have been conserved (or *vice-versa*). Features of the BAV3 E1 sequence have most recently been described by Zheng *et. al.* (1994). Comparison with E1A of Ad5 reveals significant homology between CR3, as well

as conservation of a consensus sequence important for the interaction with the p105-RB product.

The E1B region of BAV3 contains two open reading frames (ORFs), with homology to the p19 and p55 protein-coding regions of Ad5, and codes for products having predicted sizes of 17.4 and 46.7 kDa. A transcription unit having homology to that coding for pIX lies downstream of E1B of BAV3, along with regulatory sequences such as an SP1 transcriptional factor binding site and a TATA box (Zheng *et. al.*, 1994).

Since the CR3 is responsible for transactivation, the conservation of this region between Ad5 and BAV3 suggested that expression of the E1 region from one virus could potentially replace the functions of the E1 region from the other virus, and facilitate transcription of the early promoters in the genome of the other adenovirus group. This hypothesis was confirmed by coinfection with an Ad5 mutant, having deletions in E1 and E3, and a plasmid carrying the E1 region of BAV3. In the report by Zheng *et. al.* (1994), the coinfection resulted in induction of the E3 promoter of Ad5 (not removed during construction of the mutant) by E1 of BAV3, as detected by expression of a ß-galactosidase reporter gene inserted in the place of the deleted E3 coding region.

The oncogenic potential of both BAV3 and BAV8 has been demonstrated in newborn hamsters, which developed undifferentiated sarcomas at the site of inoculation (Darbyshire, 1966; Mohanty, 1971). The oncogenic nature of BAV3, as compared to the non-oncogenic serotypes BAV1 and BAV2, is consistent with the G+C content of the genome. Highly oncogenic Ads typically have

a lower G+C content than the non-oncogenic serotypes. In the case of BAVs, BAV3 has a G+C content of 48%, whereas BAV1 and BAV2 contains 62% and 61% G+C, respectively (Panigrahy *et. al.*, 1977).

The transfoming properties of BAV3, and the structural and functional similarity between its E1 region and that of Ad5, suggest that immortalization of a primary cell culture with the E1 region of BAV3, and the subsequent rescue of a BAV3 E1-deletion mutant, is possible.

2.6 Objectives of this investigation

The objectives of this study were:

(1) To construct a recombinant plasmid containing sequences from the left end of the BAV3 genome, linked to a drug-resistance marker for easy selection.

(2) To establish cell lines containing the construct from (1) inserted into the cellular genome, as candidates for the rescue of BAV mutants.

(3) To assess any morphological changes which might result from expression of the BAV3 sequences.

(4) To evaluate the utility of any novel cell lines for further research involving bovine viruses.

CHAPTER 3: MATERIALS AND METHODS

3.1 Bacterial strains

Escherichia coli (*E. coli*) strain DH5-alpha (Gibco BRL) was used for all recombinant plasmid engineering.

3.1.1 Propagation and maintenance of bacteria cultures

Bacteria cultures were grown in Luria broth [LB:10% Bacto-tryptone (Difco); 5% Bacto-yeast extract (Difco); 10% NaCl, pH 7]. Cells were stored at 4°C on LB plates, or in 15% glycerol at -20°C. Selection for antibiotic resistance was carried out in either 50µg/ml ampicillin, or 100µg/ml kanamycin, or a combination of both.

3.2 DNA ligations

Ligations were carried out at 16°C overnight (blunt ends) or at room temperature for 2-3 hours (overhanging ends). Ligations were performed in a ligation buffer supplied by NEB (10mM MgCl2; 20mM dithiothreitol; 50mM Tris; 1mM ATP, pH 7.5), using recombinant T4 DNA ligase (NEB).

3.2.1 Transformation of competent bacteria

Transformations were carried out as described in Sambrook *et. al.* (1989). E. coli DH5-alpha cultures were grown to an OD_{600} of 0.3-0.4. The culture was then aliquoted into sterile polypropylene tubes and centrifuged at 1000g (IEC Centra-7R) for 15 minutes at 4°C. The pellets were then resuspended in 25ml of ice-cold transformation buffer (75mM CaCl2; 5mM Tris-HCL, pH 7.6) and placed at 4°C overnight. The tubes were centrifuged as above, and the pellet resuspended in 1ml ice-cold transformation buffer. One hundred µl aliquots were used immediately, or stored at -70°C.

Plasmid DNA (1-10µg) was added to the competent cells and incubated on ice for 30 minutes, with occassional mixing. The cells were then heat-shocked at 42°C for 45 seconds, and diluted in 900µl SOC medium (2% Bacto-tryptone; 0.5% yeast extract; 10mM NaCl; 2.5mMKCl; 10mM MgCl2; 10mM MgSO4; 20mM glucose). One hundred µl aliquots were then plated on LB agar plates containing the appropriate antibiotic, and incubated overnight at 37°C.

3.3 Small-scale plasmid DNA preparations

Small-scale DNA isolations were carried out according to a variation of the method described by Birnboim (1983). Overnight cultures of transformed bacteria were centrifuged for 30 seconds in a microcentrifuge (12,000g). The resulting pellet was then resuspended in 100µl lysing buffer [10mM EDTA; 50mM glucose; 25mM Tris-HCl; 2mg/ml lysozyme (Sigma), pH 8.0]. The tube was incubated for 10 minutes at room temperature, followed by the

addition of 200µl alkaline-SDS (0.2N NaOH; 1.0% SDS). After incubation at room temperature for 10 minutes, 150µl of 3M NaAc (pH4.8) was added, and the sample placed on ice for 15 minutes. The sample was then centrifuged at 12,000g for 5 minutes, and the supernatant transferred to a fresh tube. The DNA was precipitated with 2 volumes of ice-cold 95% ethanol, and pelleted by centrifugation at 12,000g for 3 minutes. Following complete removal of the ethanol, the pellet was suspended in sterile water or TE buffer (10mM Tris; 1mM EDTA, pH 7.5).

3.3.1 Large-scale plasmid DNA preparations

Transformed cultures were grown overnight in 500ml of LB at 37°C. The plasmid DNA was extracted as described above for small-scale preparations, but starting with 10ml lysing buffer, and scaling up the other solutions proportionately.

3.3.2 CsCl purification of plasmid DNA

Plasmid DNA was purified by ultracentrifugation in CsCl using the procedure described in Sambrook *et. al.* (1989). DNA from a large-scale extraction was suspended in water, following the addition of ethidium bromide to a concentration of 0.8mg/ml, and CsCl to achieve a density of 1.55-1.59g/ml. The solution was centrifuged in 3ml Beckman Quick-Seal centrifuge tubes at 65,000rpm (179,000g), in a Beckman TL-100 ultramicrocentrifuge (rotor SN1533) for 20 hours at 10°C. After centrifugation, the band of supercoiled plasmid DNA was extracted with an 18 gauge needle, and transferred to an Eppendorf tube, where the ethidium bromide was removed by

extraction with isoamyl alcohol, until the DNA solution was no longer pink. The DNA was then precipitated with 2 volumes ice-cold ethanol, following a 3-fold dilution of the solution to dissolve residual CsCl.

3.3.3 Restriction enzyme digestion and gel electrophoresis

Digestions were carried out at 37°C, from 30 minutes to 16 hours, according to suppliers' specifications. Usually, 1-10µg of DNA was digested in 1x reaction buffer, supplied by the manufacturer, containing the appropriate units of restriction enzyme (NEB) and, if required, bovine serum albumin.

Digests were added to electrophoresis loading buffer (20% glycerol; 2% SDS; 0.05% bromophenol blue) and fragments were separated by agarose gel electorphoresis in 0.7-1% agarose gels, at 1-10V/cm in TAE buffer (40mM Tris; 20mM acetic acid; 1mM EDTA, pH 8.0). DNA was visualized by staining the gel with 0.5µg/ml EtBr, for 10-30 minutes, followed by illumination on a UV transilluminator. Gels were photographed with a Polaroid Land camera, with Polaroid type 57 film. The sizes of the DNA fragments were estimated using a lambda DNA-HindIII digest (NEB) as a molecular weight marker.

3.4 Mammalian cell culture

Mayden-Darby Bovine Kidney cells, E61, E24 and primary bovine kidney and lung cells were maintained in Earle's Minimum Essential Medium, supplemented with 6% Donor Bovine Serum (Cansera, Rexdale, Ontario), 0.29µg/ml L-glutamine, 0.225% sodium bicarbonate and 1.5% antibiotic-antimycotic [10,000 units/ml

penicillin, 25µg/ml amphotericin B and 10,000µg streptomycin (Gibco BRL)]. Cells were maintained at 37°C at constant atmospheric pressure, in a CO_2 incubator.

3.4.1 Fetal calf tissue and primary cell culture

Primary bovine lung cells were cultured from tissue excised from a deceased fetal calf, within 1 hour of being removed from the cow (Kingma Meat Products Ltd., Wellandport, Ontario). Following removal of the lungs (as well as kidney and liver), $1cm^3$ cubes of tissue were rinsed with iodine and chopped into small pieces in a sterile culture dish using a scalpel. The tissue was then placed in an erlenmyer flask containing 1x trypsin, and stirred on a magnetic stirring plate for a total of 1 hour. Aliquots of 1ml were drawn at 15 minute intervals, and added to 60mm culture dishes. The medium was changed daily, and the establishment of a cell monolayer was monitored closely for 2 weeks.

3.5 Mammalian cell transfections

Cells were transfected using both the method of calcium phosphate precipitation and that utilizing the commercial polycationic lipid product LIPOFECTAMINE™ (Gibco BRL). A series of conditions were tried in order to optimize the transfection efficiency but, in general, the procedures were performed as follows. For the method of calcium phosphate precipitation, DNA was added to 450µl tris-EDTA buffer(pH 7.4) and 50µl 2.5M $CaCl_2$. The mixture was then added dropwise into 500µl HEPES (*N*-2 hydroxyethyl-piperazine-*N*'-2-ethanesulfonic acid) buffer (pH 7.05), while gently

bubbling the solution with a pasteur pipette. After 20 minutes at room temperature, the solution was added to the cells, without removing the medium. Cells were seeded at densities of $5x10^5$/60mm culture dish, or $1.5x10^6$/100mm culture dish, 24 hours prior to transfections. After 4-24 hours incubation, the medium was replaced.

LIPOFECTAMINE™ was used according to the manufacturer's instructions. Briefly, DNA and LIPOFECTAMINE™ were added to separate tubes of serum-free medium (MEM). The solutions were then gently mixed together, and allowed to sit at room temperature from 15-45 minutes, after which they were added to cells which had been washed once with serum-free medium. After 2-6 hours, complete medium was added to the dish, which was, in turn, replaced with fresh medium 24 hours later.

3.5.1 ß-galactosidase assays

DNA uptake was assayed using the reporter plasmid construct pCMV-ßgal, carrying the structural gene for ß-galactosidase, under control of the cytomegalovirus (CMV) promoter. Forty-eight hours after transfection, cell monolayers were fixed for 5 minutes in 4% paraformaldehyde, pH 7.4. Fixative was then removed by rinsing 3 times with 1X phosphate-buffered saline (PBS), the second rinse lasting 10 minutes. The chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was then added at a concentration of 1 mg/ml, in a solution of 35mM $K_3Fe(CN)_6$, 35mM $K_4Fe(CN)_6$, 1mM $MgCl_2$, in PBS. The cells were then incubated at 37 degrees for 2-24 hours, after which time cells expressing ß-gal appeared blue.

3.6 Microscopy

Cell counts for ß-gal assays were performed under 100x magnification, on a Telaval 3 microscope (Carl Zeiss, West Germany). Stained cells were photographed on a MC63 photo-micrographic camera, mounted on a DRC stereo microscope (Carl Zeiss, West Germany), under 100x magnification. Non-adherent cells were photographed by phase-contrast microscopy, using a phase-contrast objective, 250x magnification.

3.7 Extraction of mammalian genomic DNA

Cells were lysed in 10mM tris, 10mM EDTA, 0.4% SDS, 50 ul/ug *pronase* (Boehringer Mannheim), for 90 minutes at 37°C. The lysate was then extracted three times with phenol/choroform, following precipitation of DNA with 2 volumes ice-cold ethanol. The DNA was resuspended in H_2O, and checked for purity by spectrophotometry at A_{260} and A_{260}/A_{280}.

3.8 Southern blotting and hybridization

Genomic inserts were detected by the method of Southern (1975) as modified by Ausubel *et. al.* (1992).

3.8.1 Transfer of DNA to nylon membrane

Genomic DNA was digested for 4-6 hours, followed by the addition of loading buffer (5µl/20µl digest). The samples were then separated by agarose gel electrophoresis (0.8% agarose). Following electrophoresis, the gel was stained with 0.5µg/ml EtBr and photographed on a UV light source. The gel was then placed in

250mM HCl for 8 minutes, with gentle agitation. Following a brief rinse in deionized water, the gel was immersed in 1.5M NaCl/0.5M NaOH, for 2x15 minutes, with gentle rocking. During this step, a nylon membrane (S&S), which was cut to fit the size of the gel, was prepared by floating in deionized water until saturated, then placed in 0.5M NaOH for 5 minutes. A transfer container was set up by placing a piece of 3MM Whatman paper in an electrophoresis box, bridging the 2 reservoirs. After the Whatman paper was wetted with 0.5N NaOH, the gel was inverted and placed on the paper. The nylon membrane was then placed on top of the gel, and 2 more pieces of 3MM Whatman paper placed over the membrane. A 5cm thick stack of paper towels was placed on top of the Whatman paper, along with a 0.2-0.4kg weight. After transfer was complete (overnight), the nylon membrane was dried at room temperature. The DNA was fixed to the membrane by applying UV radiation (254nm) in a UV Stratalinker 1800 (Stratagene).

3.8.2 Radiolabeling of DNA probes

Radiolabeled DNA probes were synthesized by the method of nick-translation, using a commercial nick-translation system (Promega). For 0.5µg of cesium chloride purified template DNA in a 25µl reaction, the following components were added: 2.5µl 10x nick-translation buffer; 5µl nucleotide mix (300µM stock); 35µCi α^{32}P-dATP; 2.5µl DNA polymerase I/Dnase I mix; nuclease-free water to 25µl. The reaction was then incubated at 15°C for 1 hour, after which stop-solution (provided) was added. Prior to use, the

reaction tube containing the probe was placed in boiling water for 2 minutes.

3.8.3 Prehybridization, hybridization and washing

The prehybridization, hybridization and washing was performed at 68°C, in a Hybaid hybridization oven. Prior to hybridization, the membrane was conditioned in a solution containing 6xSSC, 5xDenhardt's reagent [50xDenhardt's reagent: 1% Ficoll (Pharmacia); 1% polyvinylpyrrolidone; 1% bovine serum albumin (Sigma)]; 0.5% SDS and 100µg denatured herring sperm DNA (Gibco BRL), for 1 hour. Hybridization was performed overnight in a solution of 6xSSC, 0.5% SDS, 100µg denatured herring sperm DNA and labelled probe (approx. $0.2x10^8$ cpm). The membrane was washed, following hybridization, for 15 minutes in 6xSSC, 0.5% SDS. The membrane was then washed a second time in 1xSSC, 0.5% SDS, for 15 minutes, followed by a third was in 0.1XSSC, 0.5% SDS, for 30 minutes.

The membrane was placed on a piece of 3MM Whatman paper, in an X-ray cassette containing a Cronex intensifying screen, covered with plastic wrap and exposed to Kodak X-OMAT X-ray film for at least 6 hours.

3.9 Extraction of cellular RNA

Mammalian cultures were lysed directly in the culture dish by adding 1ml TRIZOL™ reagent (Gibco BRL) to a 60mm dish. The homogenate was transferred to a 1.5ml microcentrifuge tube, and incubated for 5 minutes at room temperature. A volume of 0.2ml of

chloroform was then added, followed by vigorous shaking for 15 seconds, and 2-3 minutes incubation at room temperature. The samples were then centrifuged at 12,000xg for 15 minutes. Following centrifugation, the upper, aqueous phase was transferred to a fresh tube, mixed with 0.5ml isopropyl alcohol, and incubated at room temperature for 10 minutes. The samples was then centrifuged for 10 minutes at 12,000xg. Following removal of the supernatant, the pellet was washed once with 75% ethanol, and centrifuged at 7,500xg for 5 minutes. The supernatant was once again removed, and the RNA was then stored as a precipitate in 70% ethanol, or suspended in H_2O and used immediately.

3.9.1 Northern blot and hybridization

Total RNA (30μg) was separated by electrophoresis in a 1% agarose-formaldehyde gel [1% agarose; 1x (20mM) MOPS (3-(N-morpholino) propanesulfonic acid); diethyl pyrocarbonate (DEPC)-treated H_2O; 0.66M formaldehyde], for 4 hours at 0.5V/cm, using an electrophoresis buffer of 10xMOPS/EDTA (0.2M MOPS; 50mM sodium acetate; 10mM EDTA, pH 7.0). Prior to loading on the gel, the RNA samples were added to electrophoresis sample buffer (0.75ml formamide; 0.15ml 10x MOPS; 0.24ml formaldegyde; 0.1ml deionized DEPC-treated water; 0.1ml glycerol; 0.08 ml 10% (w/v) bromophenol blue). The RNA was transfered to a nylon membrane as described for the Southern analysis above, but without soaking in HCl and NaOH/NaCl,.and using 10xSSC as the transfer buffer. Prehybridization, hybridization and washing were performed as previously described.

3.10 Immunoprecipitation of cellular proteins

Cells were plated at densities of $5x10^5$/60mm culture dish, 24 hours prior to immunoprecipitation assays. The medium was then replaced with methione-free medium (ICN) supplemented with L-glutamine [*glutamax*™ (Gibco BRL)]. After 1 hour, the cells were labelled with ^{35}S-methionine, which was added to the culture dish at a concentration of 100 mCi/ml. The cells were incubated for 2 hours, after which the medium was removed. The cells were then lysed by addition of 1 ml RIPA buffer, containing the protease inhibitor *aprotinin*™ (Sigma), for 10 minutes at 4°C. Cellular debris was pelleted by microcentrifugation at 10,000g for 5 minutes, and the supernatant transferred to a fresh tube. Rabbit polyclonal antibodies, raised to the carboxy terminus of the 12S E1A product of Ad2, and conjugated to agarose-protein A beads (Santa Cruz Biotechnology, Santa Cruz, California), were then added, and the samples placed on a rocking platform for 2 hours at 4°C. Immunoprecipitate complexes were then collected by centrifugation at 10,000g for 20 seconds, followed by removal of the supernatant and washing with 1 ml ice-cold RIPA buffer. Centrifugation and washing was repeated 3 more times, after which final traces of buffer were removed with a pasteur pipette which had been narrowed at the end in a flame. The pellets were then suspended in 50 ml of sample buffer (ß-mercaptoethanol; glycerol), and stored at -20°C until use. Immunoprecipitates were analysed by SDS-PAGE, in a 10% polyacrylamide gel, including a low-molecular weight size marker (BioRad). Prior to loading, the samples were denatured in

boiling water for 5 minutes. Gels were soaked in 1M sodium salicylate for 30 minutes, dried, and exposed to Kodak X-ray film for 10 days.

CHAPTER 4: RESULTS

Two novel bovine cell lines were established over a series of steps. First, the recombinant plasmid pKC-*neo* was constructed. This plasmid was then used to transfect both MDBK and primary cells, after which selection in G418 identified candidate cell lines. Two cell lines, E61 and E24, were analysed for DNA content, revealing the presence of the pKC-*neo* sequences in each of the cellular genomes. The E61 cells seemed to be expressing E1 RNA transcripts, according to Northern analysis. In addition, both cell types were examined for the ability to take up exogenous DNA, at which the E24 line was found to be particularly efficient.

Although conditions for primary bovine kidney cells were optimized, these cells were not used in the final experiments, due to problems with maintaining a consistent culture.

4.1 Construction of recombinant plasmid pKC-*neo*

The plasmid pKC-*neo* was constructed according to the following strategy, starting with pSV2-PK, pSV2-*neo*(mcs), and pKC19, as illustrated in figures 9 and 10. Briefly, the first step in the construction of pKC-*neo* was the conversion of the unique *Pvu*II site in pSV2-PK, to *Kpn*I, by digestion of pSV2-PK with *Pvu*II, followed

by addition of *Kpn*I linkers and religation. The *neo*^{r} gene was then taken from pSV2-*neo*(mcs) by digestion with *Bam*HI and *Hin*dIII, and placed between these same sites in pSV2-KK, to generate pSV2-*neo*K.

The SV40 promoter and *neo*^{r} gene were then removed from pSV2-*neo*K by digestion with *Kpn*I, and inserted into the unique *Kpn*I site in pKC19. The resulting plasmid, pKC-*neo* , containing the left 19.7 % of the BAV3 genome and the *neo*^{r} gene from Tn5, was confirmed by digestion with several informative restriction enzymes, followed by separation of the fragments on a 0.9% agarose gel (figure 11). Note that the values for all restriction site positions, indicated in bp, are approximations.

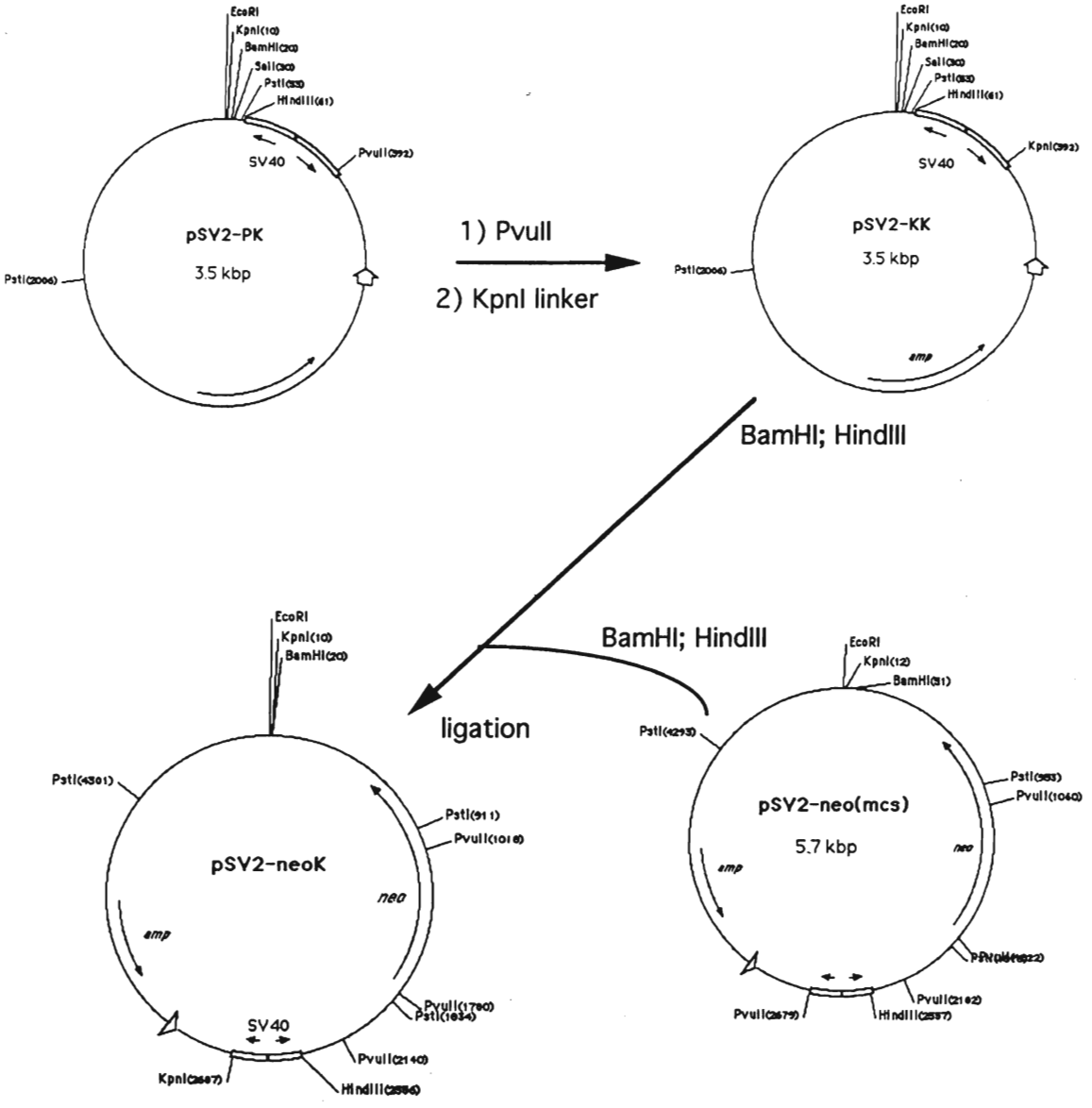


Figure 9. Step 1 in the construction of plasmid pKC-*neo*. The unique *Pvu*II site in pSV2-PK was converted to a *Kpn*I site, generating pSV2-KK, after which the neomycin-resistance gene from pSV2-*neo*(mcs) was inserted between the *Hin*dIII and *Bam*HI sites of pSV2-KK, to generate pSV2-*neo*K.

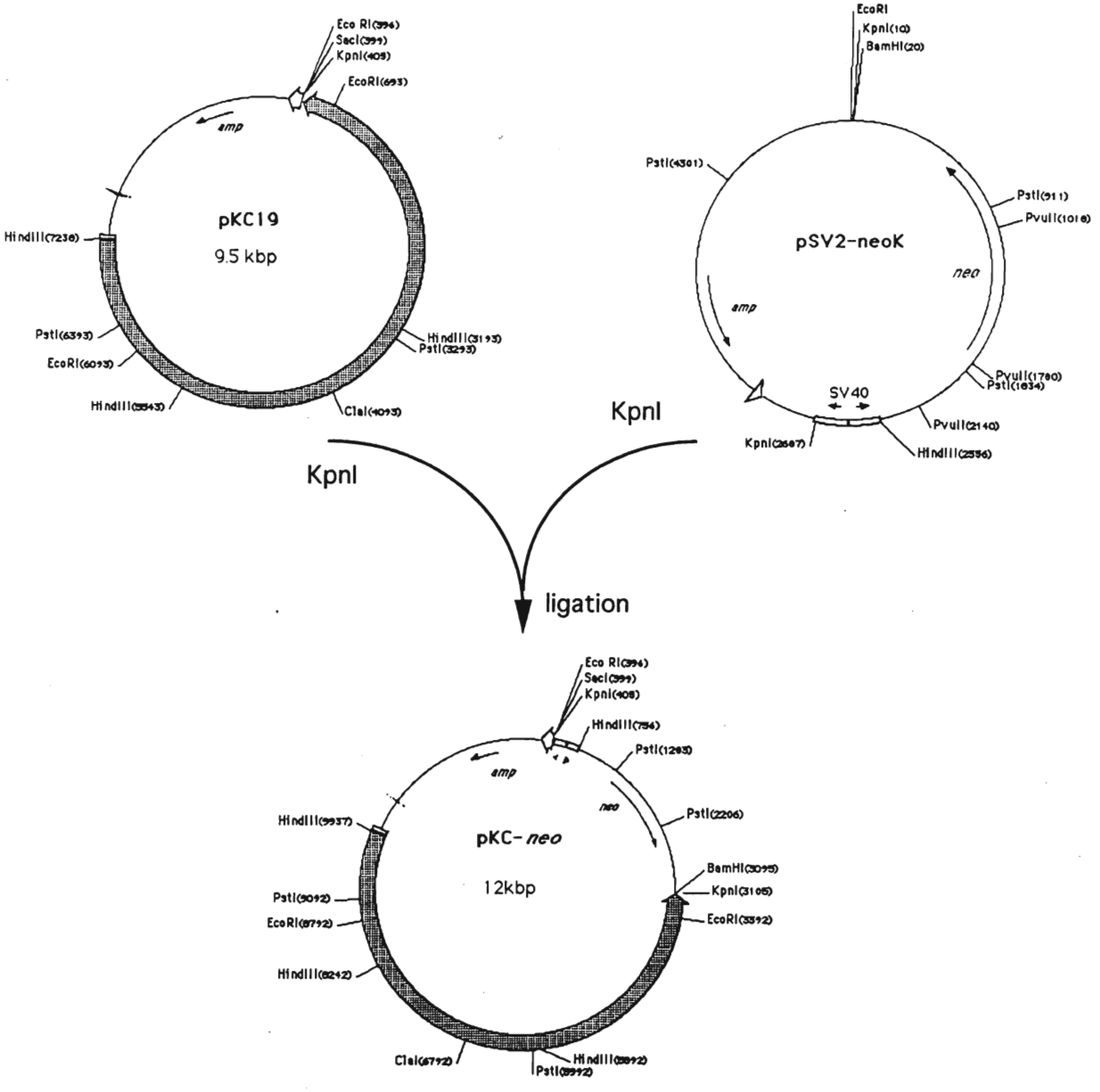


Figure 10. Step 2 in the construction of plasmid pKC-*neo*. The SV40 and neomycin-resistance sequences were removed from pSV2-*neo*K by digestion with *Kpn*I, and inserted into the unique *Kpn*I site of plasmid pKC19.

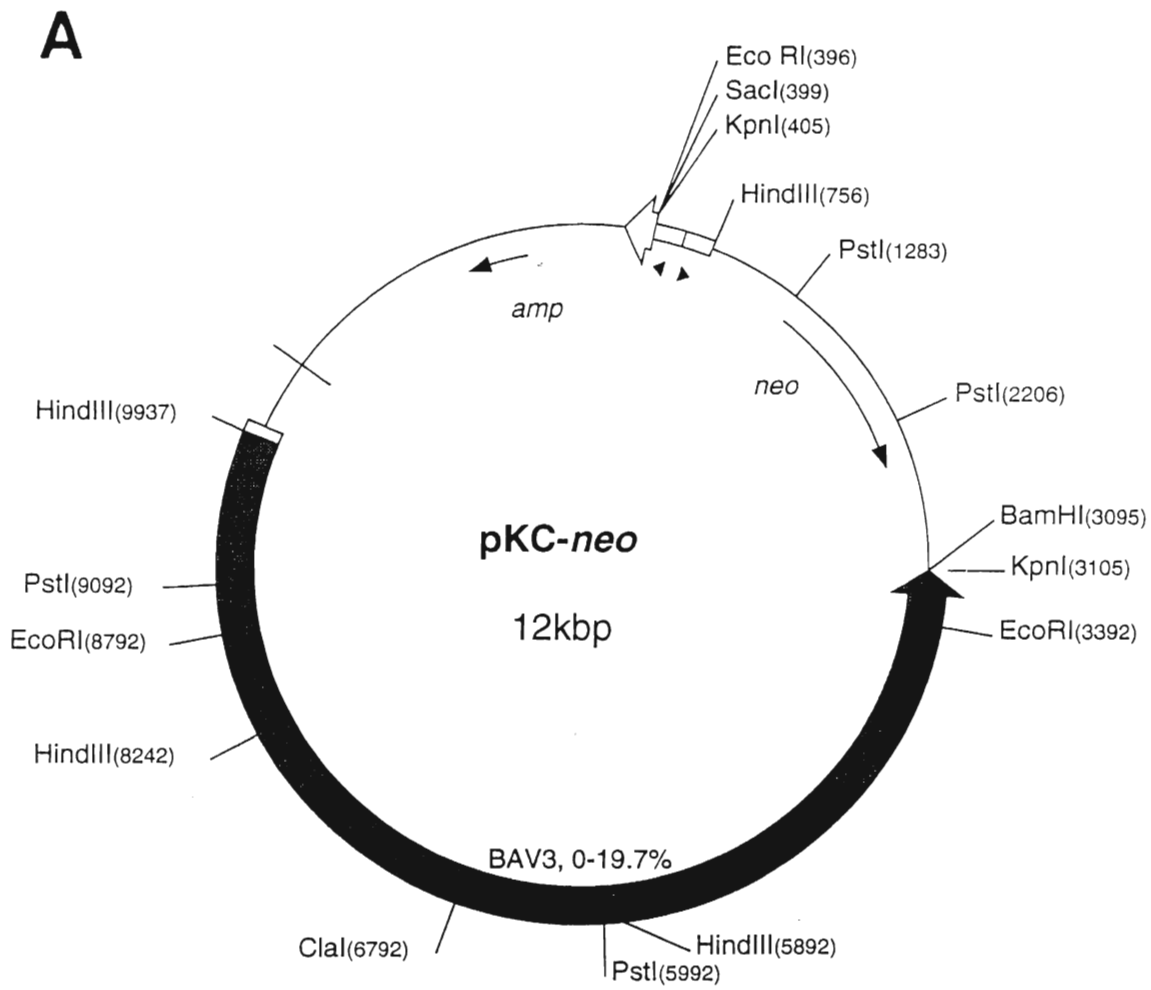


Figure 11. Recombinant plasmid pKC-*neo*. (A) Restriction map of pKC-*neo*. The shaded region represents the left 0-19.7% of the BAV3 genome. (B) Agarose gel analysis of restriction fragments of pKC-*neo*, generated by digestion with BamHI, EcoRI, HindIII, KpnI, ClaI and PstI, lanes 2-7, respectively. Lane M contains a lambda marker (*Hin*dIII digest).

4.2 Establishment of primary lung cell culture

Two weeks following disaggregation of male fetal calf tissue, cultures of kidney and lung cells were established. Problems with maintaining the culture of kidney cells, however, resulted in the eventual termination of their use. The primary lung fibroblasts are presented in the photographs in figure 12.

4.3 Optimization of transfection conditions

Prior to experimental transfections, the optimum conditions for both methods, the calcium phosphate precipitation and the polycationic lipid LIPOFECTAMINE™, needed to be established, not only to identify the optimum conditions, but also to choose the most effective method (figure 13). The calcium phosphate method was optimized in terms of DNA load in the precipitation event, a critical parameter as described by Loyter *et. al.* (1982), whereas transfection with LIPOFECTAMINE™ was optimized in terms of LIPOFECTAMINE™ volume per culture dish, with toxicity being the limiting factor and, therefore of major consideration.

The method of calcium phosphate precipitation was optimized in terms of DNA load, or the amount of DNA present in the precipitation reaction, prior to addition to the culture dish. The choice of 10, 20 and 30µg of total DNA was based on results by Loyter *et. al.* (1982), which showed the optimum DNA load for murine cells to lie at 20µg/ml $CaPO_4$.

According to manufacturer's specifications, the optimum amount of DNA within the tested range for LIPOFECTAMINE™ was 2µg for a 35mm dish of NIH3T3 cells of 80% confluency, using 9µl of lipofectamine. The ratio of 4.5µl LIPOFECTAMINE™ to 1µg plasmid DNA was therefore chosen for the assay to determine the optimum amount of LIPOFECTAMINE™ for applications on the MDBK, primary lung and primary kidney cells. The optimum amounts of LIPOFECTAMINE™ and DNA were scaled up accordingly by surface area, to accomodate the larger experimental culture dishes.

It was determined that LIPOFECTAMINE™ was the most effective method of DNA transfection, for all cell types, and that 20-40µl LIPOFECTAMINE™/60mm culture dish was a sufficient amount for efficienct DNA delivery, while keeping toxicity at a minimum.

Each assay dish received an equal amount of reporter plasmid DNA (2µg/35mm dish). For the method of calcium phosphate precipitation, the total amount of DNA was adjusted using herring sperm DNA (Gibco).

4.4 Dose-response for selection in G418

The minimum concentration of G418 required to eliminate non-resistant cells within 10 days was determined for each cell type (figure 14). Based on these results, 800µg/ml and 400µg/ml G418 was used to select resistant MDBK and primary lung cells, respectively.

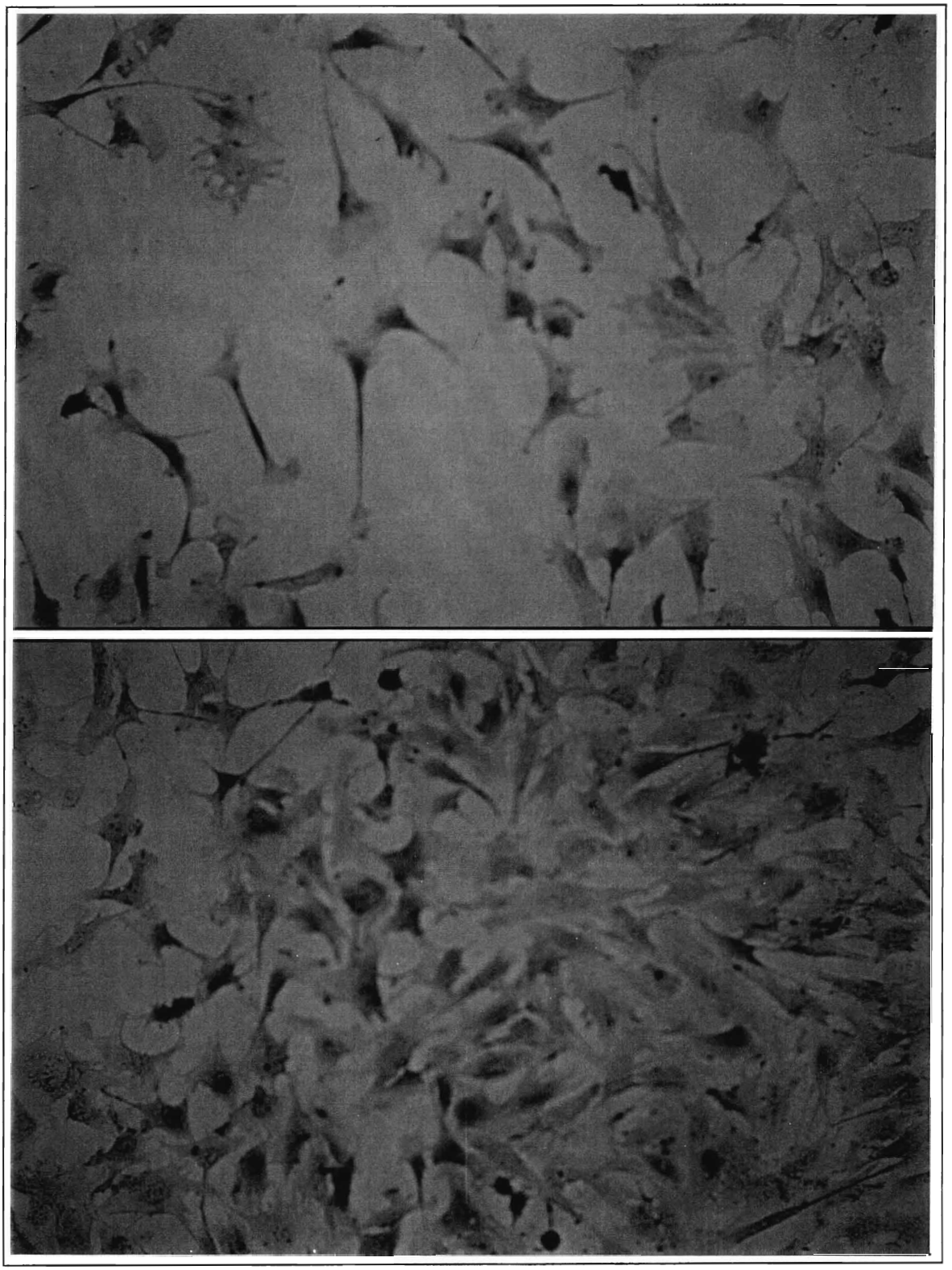
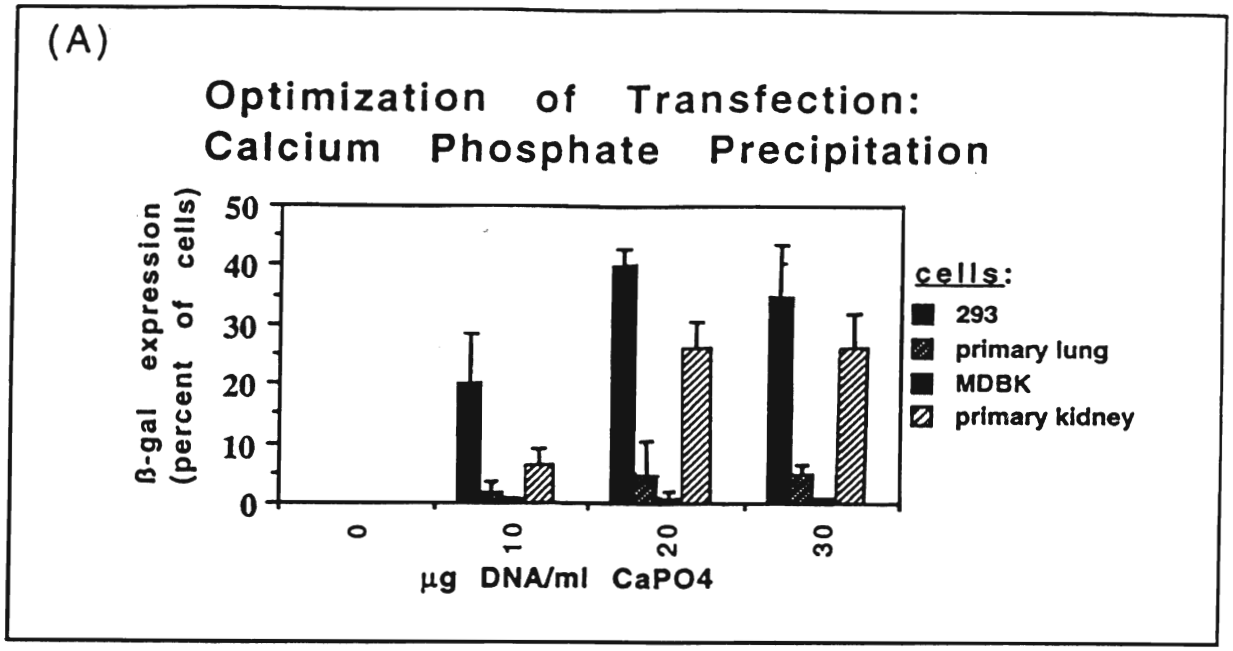


Figure 12. Primary bovine lung fibroblasts, cultured from fetal bovine lung tissue (100x magnification).



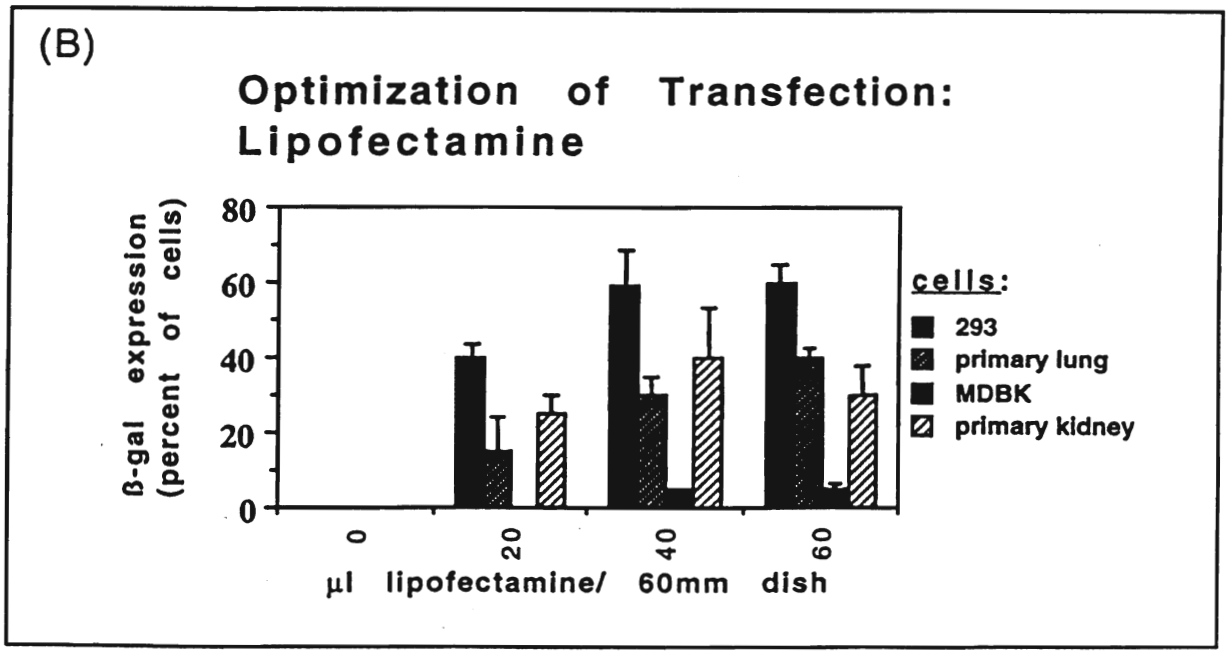


Figure 13. Optimization of transfection. Cells were transfected with pCMV-βgal, using calcium phosphate precipitation (A) and lipofectamine (B). Efficiency of DNA uptake was expressed as percentage of total cells expressing β-gal (blue in presence of Xgal).

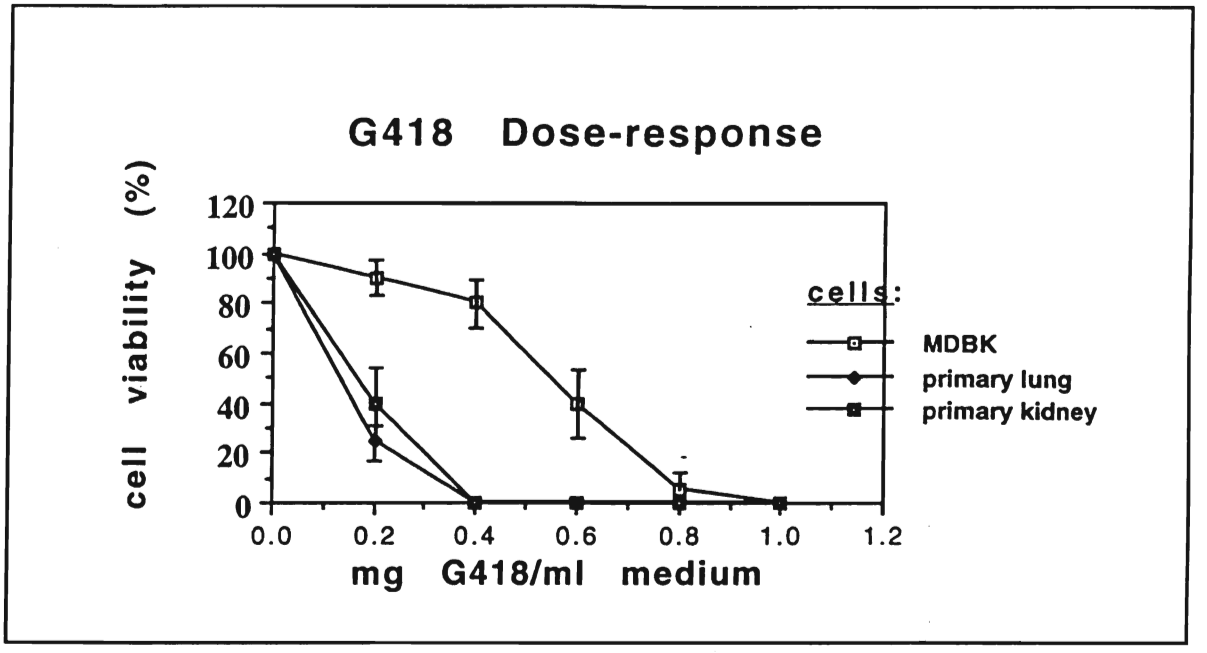


Figure 14. Dose-response curve for G418 cytotoxicity. Cells were seeded in triplicate at a density of $2x10^4$/35mm well. Response to G418 was assayed by cell counts after 10 days exposure to G418, which were then expressed as percentage of control counts, to accomodate differences in density requirements for each cell type.

4.5 Establishment and analysis of E61 cells

After three weeks under selection, all transfected MDBK cells in all experimental and control plates died, except for those contained in a single focus in dish E6. This focus was lifted from the dish by the use of a sterile ring filled with versene (5 mM EDTA, 30mM NaCl, KCl, Na_2HPO_4, KH_2PO_4, 10 mM glucose, H_2O), and transferred to a fresh, 15mm well. The cells were designated E61, and were eventually expanded into progressively larger culture dishes, and maintained under 400µg/ml G418 throughout the experiments.

The E61 genomic DNA was analysed by Southern hybridization (figure 15). The DNA was digested by *Hin*dIII, then probed with the nick-translation products of pKC-*neo*, labelled with ^{32}P-dATP. As controls, *Hin*dIII digests of pKC-*neo* and MDBK DNA were also probed on the same membrane. The hybridization pattern resulting from the pKC-*neo* digest was as expected, producing the 4 characteristic bands of the *Hin*dIII digest. The probe did not hybridize to the control MDBK digest.

Hybridization to several bands in the E61-digest lane revealed the presence of an insert of pKC-*neo* into the E61 genome. The pattern of hybridization to the E61 digest indicated that the integrity of the E1 region was maintained, as illustrated in figure 15(C), where the relevent *Hin*dIII fragments, which produce signals in the hybridization of figure 8B, are shown relative to a linear map of the plasmid.

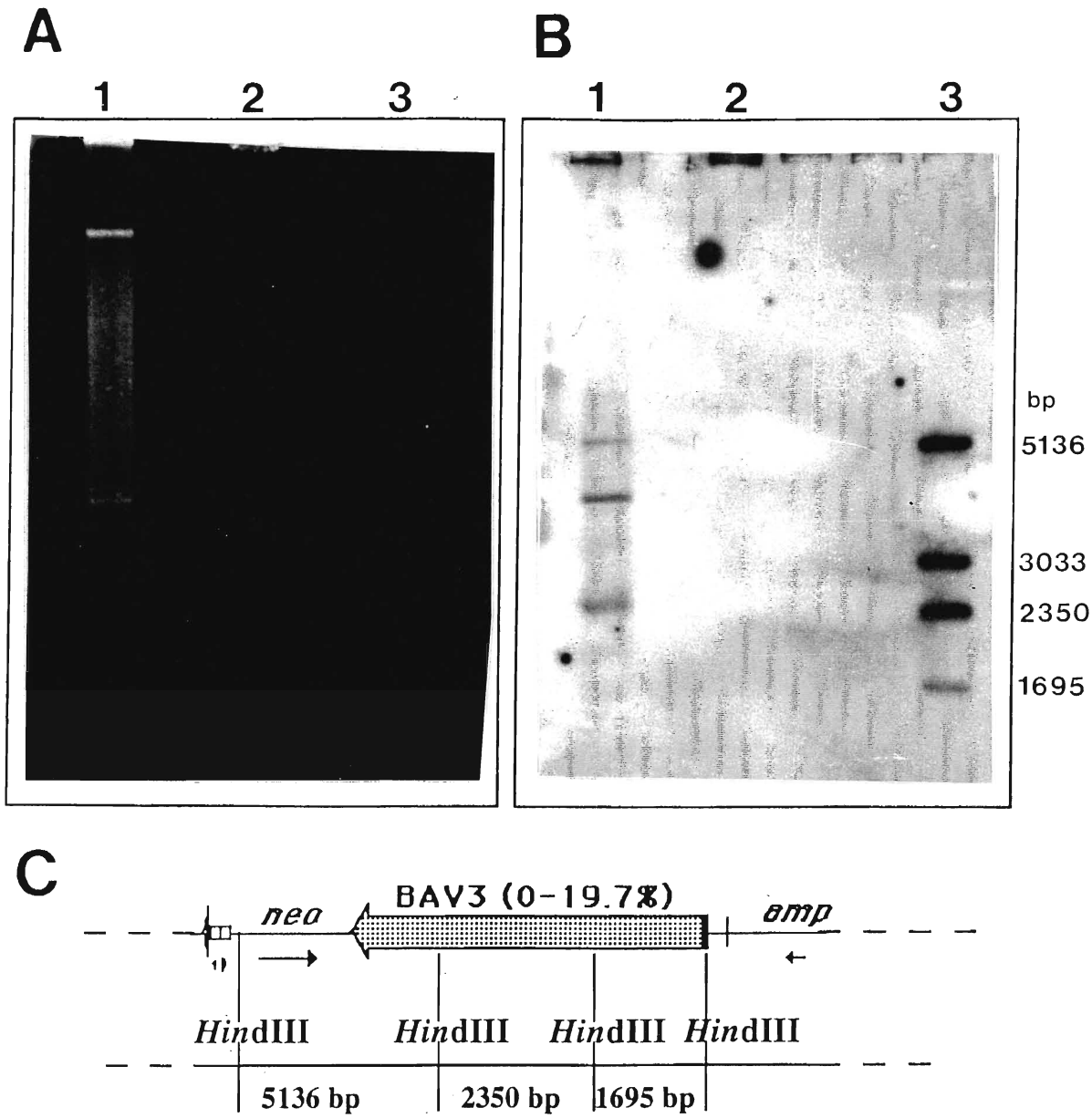


Figure 15. (A) Agarose gel electrophoresis of E61 and MDBK DNA. Lanes 1 and 2 contain 20μg of E61 and MDBK genomic DNA, respectively, digested with *Hin*dIII. Lane 3 contains 1ng of pKC-*neo*, digested with *Hin*dIII. (B) Southern transfer of (A), followed by hybridization with nick-translated ^{32}P-labelled pKC-*neo*. (C) Schematic diagram corresponding to the possible orientation of the pKC-*neo* insert in the E61 genome.

Starting from the left-end of the BAV3 genome (0%), the fragments encompassing the BAV3 and SV40-neomycin-resistant sequences have approximate lengths of 1695, 2350 and 5136bp, and produced signals at these positions on the hybridization membrane. Additional signals were produced at approximately 2000, 3500, 4000, 6000 and 7000bp. These signals were faint, relative to the bands at 2350 and 5136bp, except for the band at approximately 4000bp, which had equal intensity. The presence of at least two of these bands could be explained by *Hin*dIII sites in flanking genomic regions. The remainder may be due to methylation of sites in a subpopulation of the cells.

The RNA content of the E61 cells was analysed by Northern hybridization of total cellular RNA (figure 16). The plasmid pKC19 was used as a probe, instead of pKC-*neo*, since pKC19 does not contain the *neo*^{r} sequences, and would therefore not detect the transcripts from that region. The probe hybridized to the RNA from the E61 and BAV3-infected MDBK cells (lanes 1 and 3, respectively), but not to the RNA from the MDBK cells (lane 2).

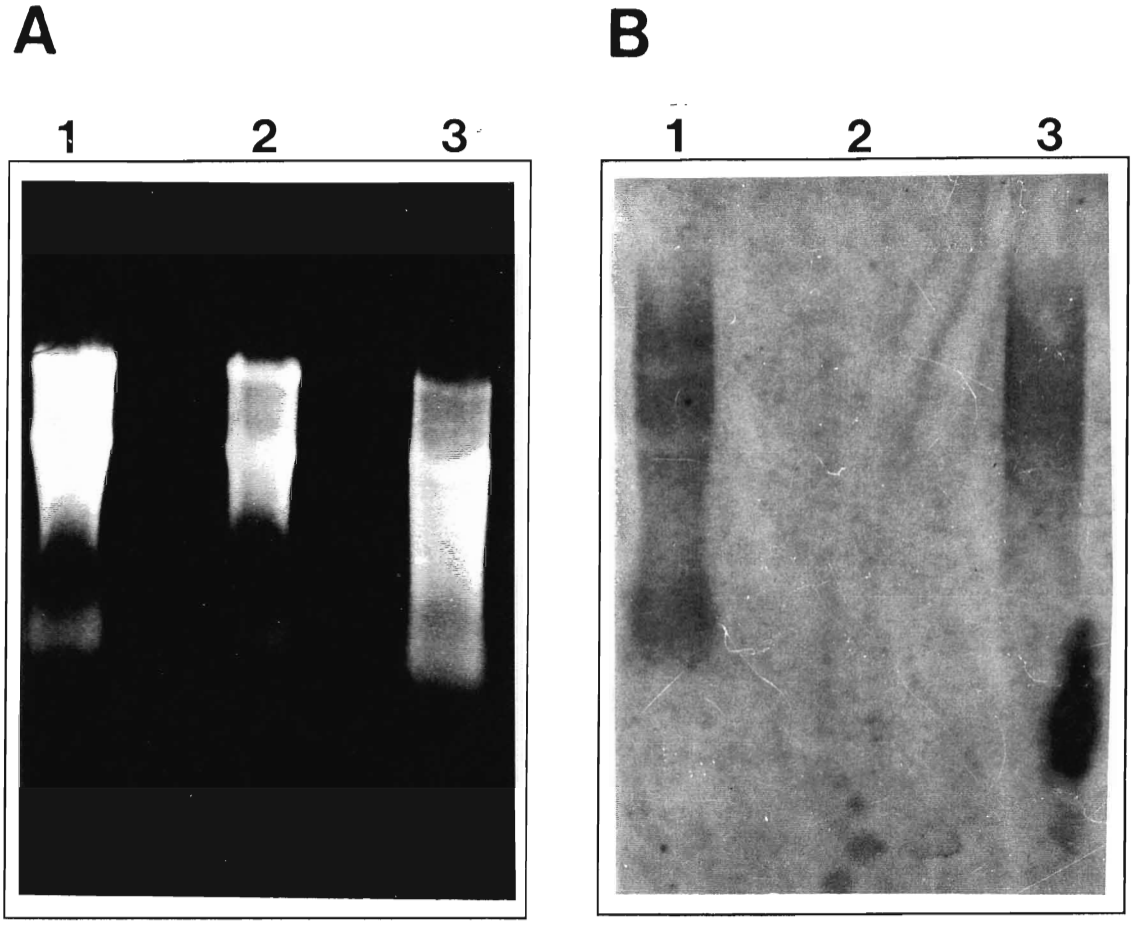


Figure 16. (A) Agarose gel electrophoresis of E61 and MDBK RNA. Lanes 1 and 2 contain 30µg total RNA from E61 and MDBK cells, respectively. Lane 3 contains 30µg total RNA from BAV3-infected MDBK cells, 8 hours post-infection. (B) Northern transfer of (A), followed by hybridization with nick-translated ^{32}P-labelled pKC19.

4.6 Establishment and analysis of E24 cells

All of the primary lung cells, except for a single focus, died after two weeks under selection. The single remaining focus did not appear to expand under the selection conditions and, as a result, selection was removed. The cells, designated E24, were expanded into progressively larger culture dishes, and have been passaged 29 times, to date.

Southern analysis of the genomic DNA content of the E24 cells is shown in figure 17. The genomic DNA was digested with *Hin*dIII, and probed with the nick-translation products of pKC-*neo*, labelled with ^{32}P-dATP. As controls, *Hin*dIII digests of pKC-*neo* and primary lung DNA were also probed on the same membrane. The hybridization pattern resulting from the pKC-*neo* digest was as expected, producing the 4 characteristic bands of the *Hin*dIII digest. The probe did not hybridize to the control lung digest.

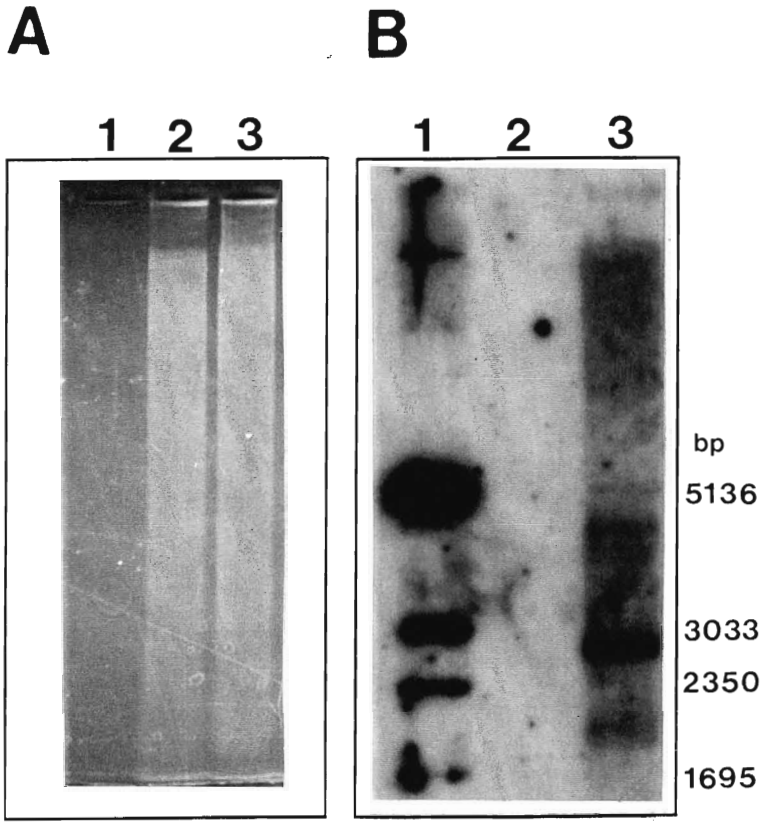


Figure 17. (A) Agarose gel electrophoresis of E24 and primary lung DNA. Lane 1 contains 1ng of pKC-*neo*, digested with *Hin*dIII. Lanes 2 and 3 contain 20μg of primary lung and E24 genomic DNA, respectively, digested with *Hin*dIII.(B) Southern transfer of (A), followed by hybridizaiton with nick-translated ^{32}P-labelled pKC-*neo*. Approximate fragment sizes are indicated in base-pairs (bp).

4.7 Immunoprecipitation

As an alternative to Northern analysis of E24 cells, cellular lysates of E24, E61, primary lung, MDBK, *293*, and both BAV2 and BAV3-infected MDBK cells were immunoprecipitated with 0.5μg of anti-Ad2 E1A rabbit polyclonal antibodies. Immunoprecipitation of E1 products from the *293* cells should have produced bands ranging from 35-53kDa, with predominant bands at 45-48.5kDa and 50-52kDa (Rowe *et. al.*, 1983; Yee and Branton, 1985). SDS-PAGE and autoradiography of the immunoprecipitates, however, revealed multiple bands in all sample lanes after 10 days exposure (figure 18).

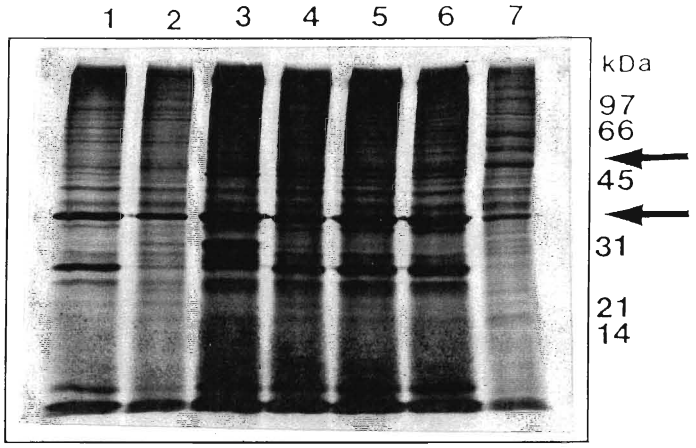


Figure 18. Immunoprecipitation of cellular proteins, using 0.5µg of anti-Ad2 E1 polyclonal antibody. Lanes 1-7: E61, E24, lung, MDBK, BAV2, BAV3, *293*. An unlabelled, low-range molecular weight standard was used for size determination (BioRad), visualized by staining in Coomassie blue R-250 dye. Expected band positions are indicated by arrows.

4.8 Morphology and growth properties of E24 cells

The E24 cells are shown in the photographs in figure19a and, at higher density, in19b. Cell monolayers were fixed and stained with a combination of methanol, formaldehyde, and crystal violet stain. The E24 cells have lost the extended processes characteristic of the fibroblastic parental primary lung cells, appearing more trapezoidal in shape. The growth of these cells seems to be limited by density factors, being unable to grow as dense foci and beyond a monolayer.

The non-adherent nature of the E24 cells is shown in phase-contrast microscopic images of E24 and primary lung cells (figure 20). The non-adherent E24 cells appear as light, rounded images, in contrast to the dark, flat adherent cells. Fibroblasts transformed by oncogenic *ras* and by E1 of Ad12 are shown for comparison.

To demonstrate the viability of the non-adherent E24 cells, 2 ml of medium was removed from a 150mm dish containing an E24 monolayer, which had been plated 2 days earlier at a density of $5x10^6$ cells, and added to a 60mm dish, along with an additional 3 ml of complete medium. The same was done for control plates of MDBK and primary lung cells. The E24 cells adhered to the culture dish and began to divide, whereas the control cells did not.

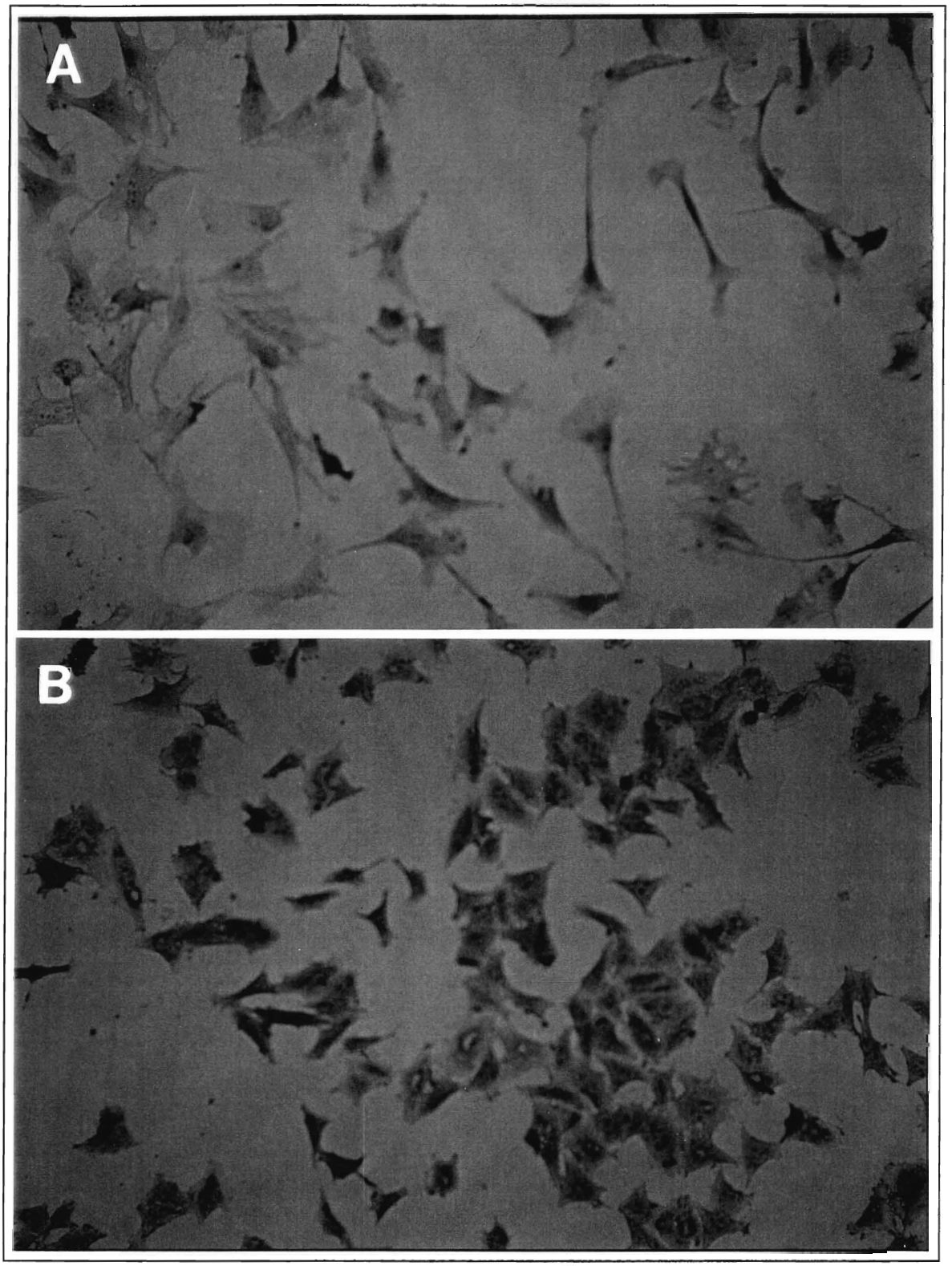


Figure 19a. Morphology of primary lung (A) and E24 (B) cells (100x magnification).

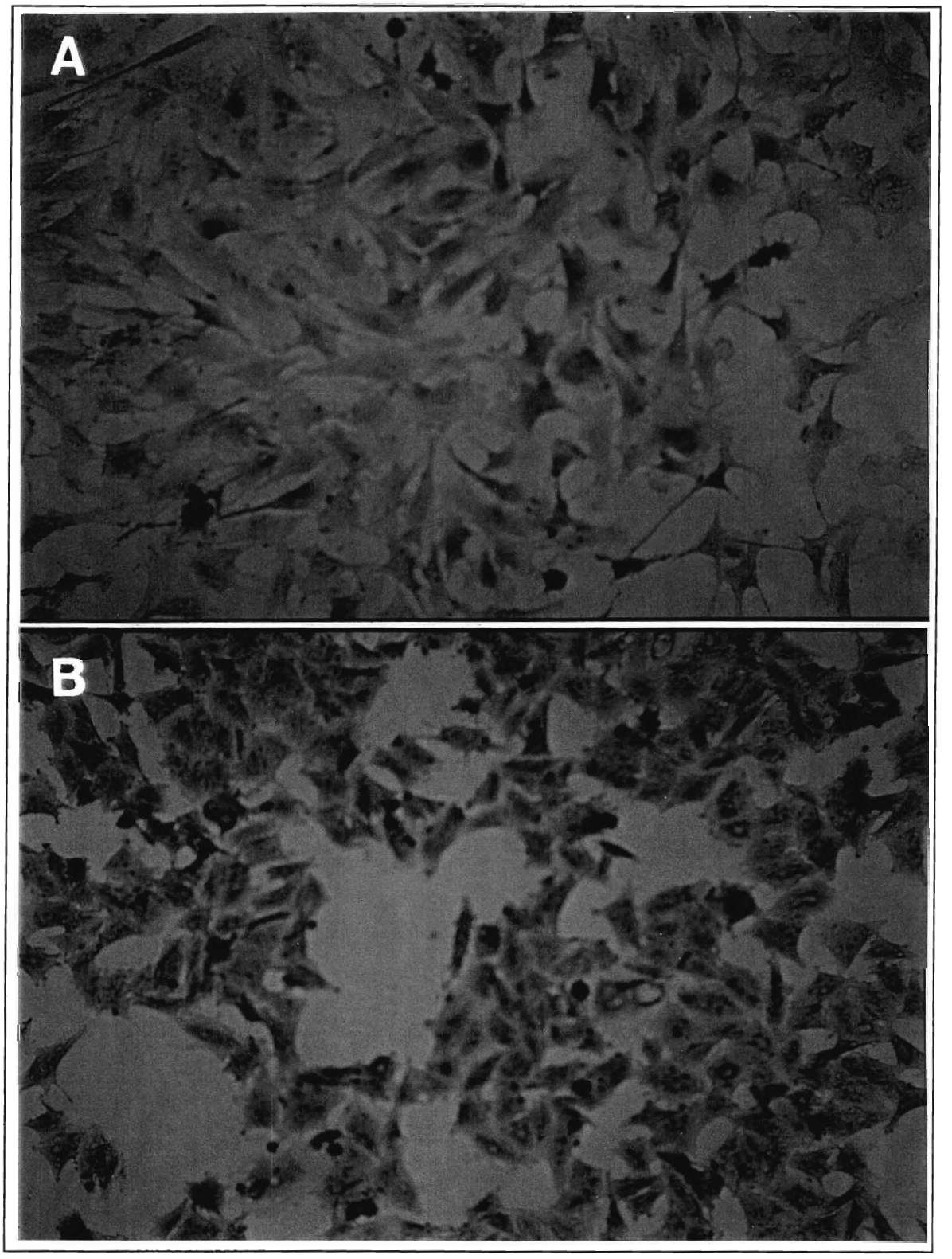


Figure 19b. Morphology of primary lung (A) and E24 (B) cells (100x magnification).

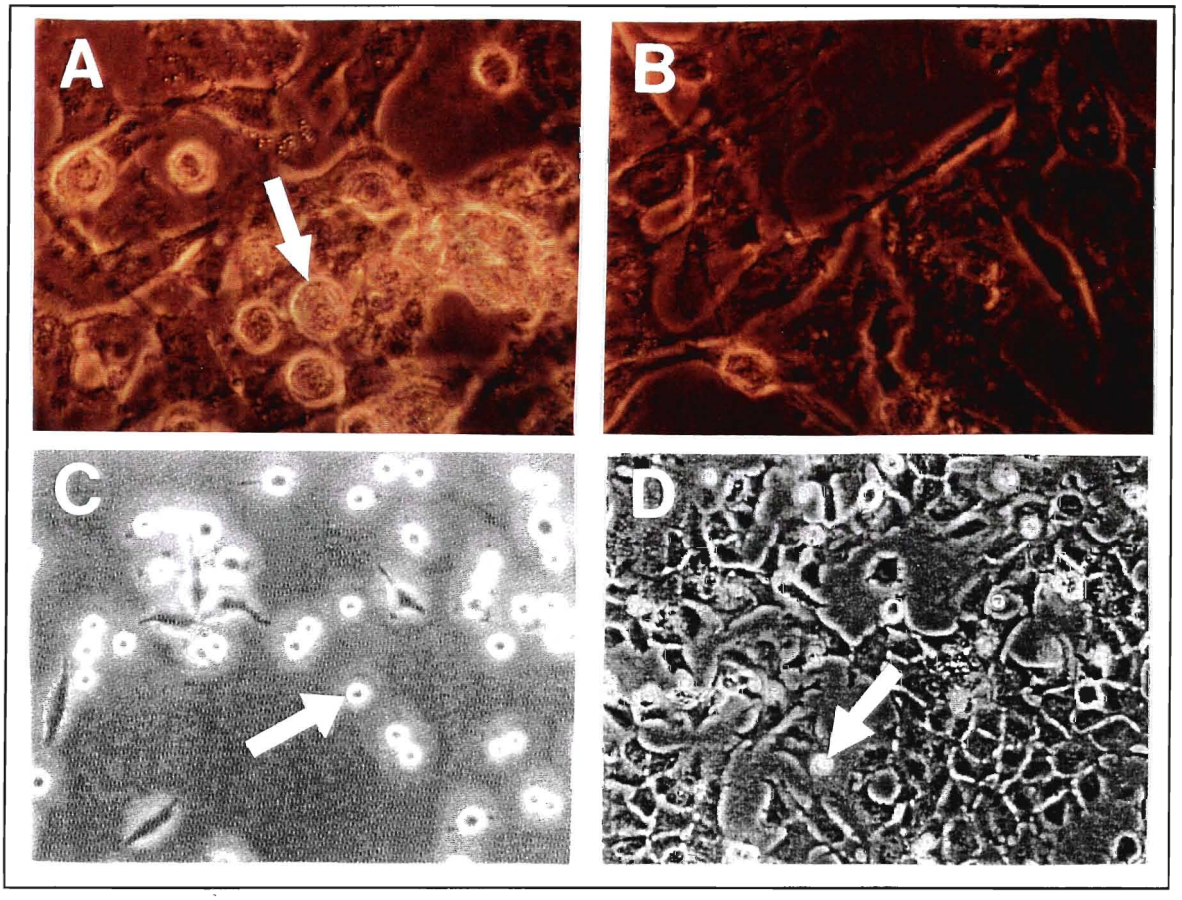


Figure 20. Non-adherent properties of E24 cells. Non-adherent E24 cells are seen as rounded images (indicated by arrows) in (A), whereas primary lung cells are shown in (B). For comparison, fibroblasts transformed with oncogenic *ras* (C) (from Saltiel, 1995) and with Ad12-E1A+p55 (D) (from White and Cipriani, 1990), are also shown.

4.9 Evaluation of DNA-uptake

The efficiency of DNA uptake for E61 and E24 cells was determined using both calcium phosphate precipitation and LIPOFECTAMINE™ (figure 21). Efficiency was calculated as the percentage of cells expressing the ß-galactosidase gene, carried on the plasmid pCMV-ßgal. Cells which had taken up and were expressing the ßgal construct turned blue in the presence of the chromogenic substrate x-gal.

As shown in panels (A) and (B) of figure 14, *293* cells exhibited the highest degree of DNA uptake (as high as 50-60%) for both transfection methods. LIPOFECTAMINE™ was the most effective method for all cell types and, using either method, the E24 cells exhibited a transfection efficiency resembling that of the parental primary cells, and much higher than that of the E61 or the MDBK cells.

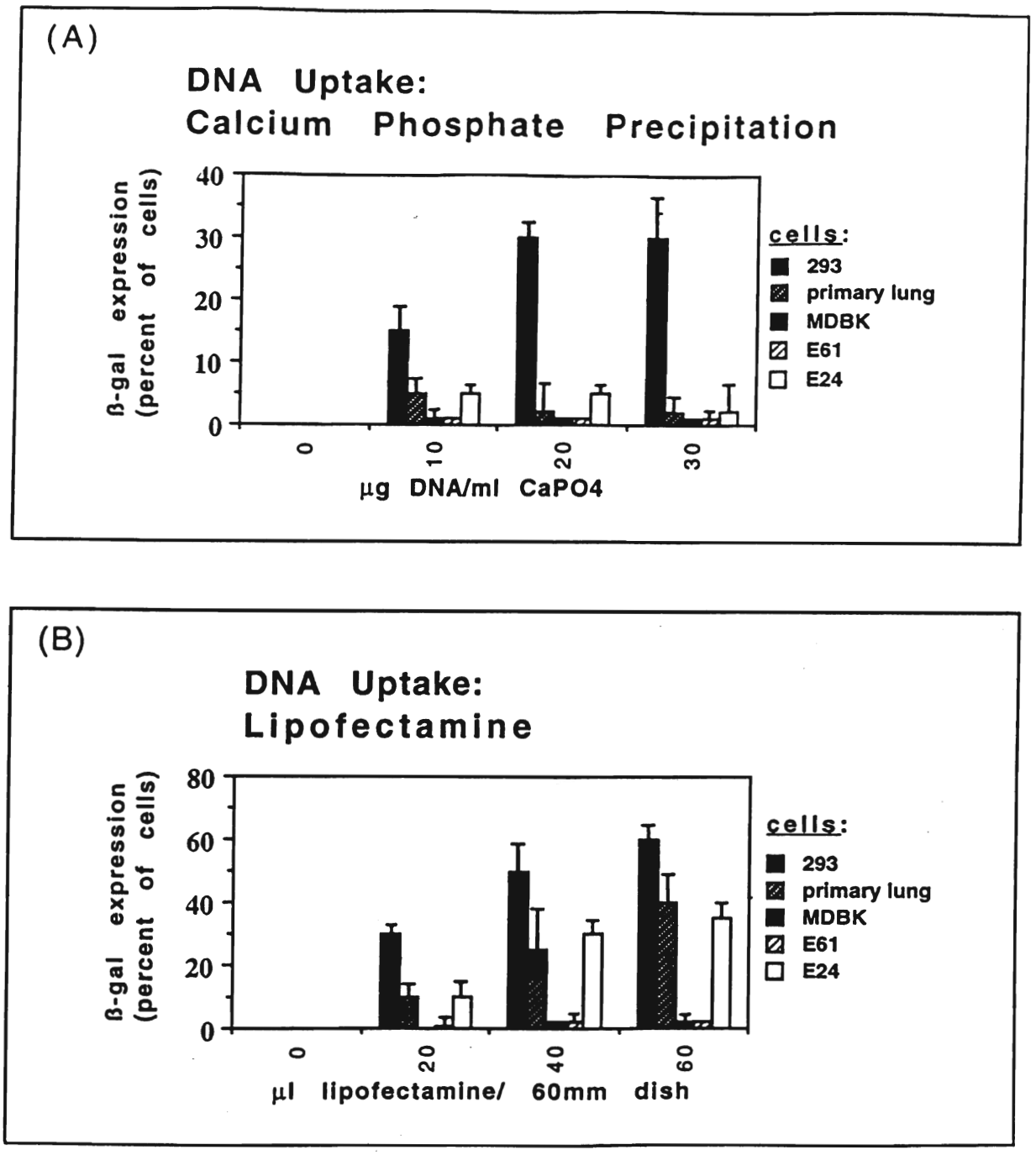


Figure 21. Evaluation of DNA uptake efficiency for E61 and E24 cells. As with previous transfection assays, ß-galactosidase expression was used to assess DNA uptake for methods using calcium phosphate precipitation (A) and lipofectamine (B). The plasmid pUC19 was used as a negative control, and did not result in a positive signal.

CHAPTER 5: DISCUSSION

Selection for candidate cell lines was based on resistance to the aminoglycoside G418. The gene encoding an aminoglycoside phosphotransferase, a G418 inhibitor, was linked to the BAV3 E1 sequences in a recombinant plasmid construct, pKC-*neo*, which was then introduced into MDBK and primary bovine lung cells. This selection system was based on the pSV2-*neo* construct of Southern and Berg (1982), which provides the advantage of positive selection for transformants due to the selective advantage conferred on the cells by the dominant drug-resistant phenotype. The approach was used as a rapid and convenient method of selecting potential E1-expressing cell lines, since integration of the G418-resistant gene into the genome of a cell would quite possibly be accompanied by the integration of the neighbouring BAV3 E1 sequences.

This pKC-*neo* construct was advantageous for the establishment of integration, since the only mammalian origin of replication, contained within the SV40 promoter sequences, requires expression of the SV40 LT antigen (as found in COS cells) for replication to commence, ensuring the absence of replication in most mammalian cells, including the ones used in this project and, in doing so, increased the likelihood of integration.

It is a reasonable assumption that integration of the complete BAV3 region contained in pKC-*neo* would result in proper expression of the E1 products, considering that the construct was made to include the upstream E1 regulatory regions, including the enhancer, CAAT box and a TATA motif, as well as the CRE, which is a target of ATF and AP-1 (Angel *et. al.*, 1987; Engel *et. al.*, 1988). Proper splicing of the E1 transcripts would be expected, since eukaryotic splicing signals have been highly conserved during evolution, and primary transcripts from different species are readily recognized and processed in heterologous mammalian cells.

Any absence of expression in cells containing an intact E1 region could possibly be the result of methylation of nucleotides in upstream regulatory sequences, since control of expression by specific patterns of methylation in these sequences has been observed in transformed cells (Kruczek and Doerfler, 1983; Doerfler, 1991). In addition, failure of the translational machinery of the cell to recognize the AUG codons in both reading frames could result in the absence of an E1B protein product, as will be discussed shortly.

The location of integration of pKC-*neo* in the E61 genome seems to have been in the region encompassing the bacterial origin of replication in the pUC19 sequences, as evident from the restriction pattern observed from the Southern hybridization. The proposed location of integration is consistent with observations that the origin of replication on M13 phage genomes are sites of recombination (Michel and Ehrlich, 1986), along with SV40 sequences which are targets for insertion and excision of the SV40 provirus (Bullock *et. al.*, 1985). These regions are locations where illegitimate recombination

has been observed, an event whereby recombination occurs through regions of very small (3-10 bp), if any, homology (Rubnitz and Subramani, 1984; Nakano *et. al.*, 1984). Such recombination events are rare, relative to homologous recombination, and are generated by enzymes normally associated with DNA replication and repair, such as DNA polymerases and topoisomerases. The origin of replication and short SV40 sequences contain targets for enzymes such as topoisomerase I, DNA gyrase, DNA polymerase and topoisomerase II, the latter of which affects illegitimate recombination between the plasmid pBR322, containing the bacterial origin of replication, and phage lambda (Michel and Ehrlich, 1986).

Recombination may be facilitated by the various enzymes even in the absence of plasmid recombination. The phenomenon of illegitimate recombination, and recombination in general, is believed to be incidental to replication and repair. To assess the applicability of these models to the insertional event in the generation of the E61 cell line, it would be worthwhile to rescue and clone the insert and the flanking genomic regions for sequence analysis at the junctions, to search for signs of a recombination event consistent with the illegitimate events observed in previous studies (for a review of illegitimate replication, see Crow and Dove, 1987).

The establishment of the E61 cells satisfied the first goal in the construction of the desired cell line. Since the E61 cells were maintained by selection in G418, it remained desirable to construct an additional cell line based on the transformation, or at least the immortalization, of primary bovine cells with the BAV3 E1 region. Such a cell line would be maintained by selection on the basis of

immortalization by E1, and would therefore remain under the pressure of maintaining the integrity of the integrated BAV3 sequences, unlike the case of the MDBK derivatives, already derived from an established cell line.

The problem with trying to establish a cell line from primary cells, however, was one of selection. If a distinctly transformed phenotype was not conferred upon the cells by the E1 region of BAV3, then the isolation of cells expressing E1 would require enough passages, perhaps 50 or more, to eliminate the non-immortalized parental primary cells. This basis for selection, however, would assume at least immortalization by E1.

Establishment of the E24 cell line was the first step in overcoming these obstacles. These cells, established from primary bovine lung cells, contain an insert which includes the BAV3 E1 region. Although the expression and integrity of the E1 region needs yet to be directly confirmed, the cells certainly exhibit a phenotype distinctly different from the parental primary lung cells. The use of positive selection to identify cells containing E1 will now allow evaluation of the resulting phenotypic changes, assuming they were conferred by expression of E1, and subsequently permit the design of selective conditions for the establishment of a cell line from primary cells, without the need for a drug-resistance marker.

The most striking morphological change in the E24 cells, when compared to the primary parental line, is the elimination of the extended processes characteristic of primary fibroblasts, and the reduction in focal adhesion events, particularly apparent in the extensive prevalence of non-adherent cells, which resemble *ras*-

transformed cells. If, in fact, the E1 sequences are being expressed in these cells, the role of p19 of E1B in the disruption of intermediate filament networks must be considered (White and Cipriani, 1989, 1990). As a result of this disruption, the detachment of the intermediate filaments from the plasma membrane may alter the organization of the cell surface, affecting cell-cell attachment and growth regulation, changes which can promote anchorage-independent growth and tumorigenicity (White and Cipriani, 1989).

Alternatively, based on similar phenotypes conferred by *ras* (Ruley, 1983) and another membrane-associated kinase oncogene, $pp60^{v\text{-}src}$ (Shalloway *et. al.*, 1987), the disruption of the intermediate filaments by p19 could alter the organization of the plasma membrane or structural elements of the cytoplasm in such a way as to affect signal transduction pathways transmitting messages from the cell surface to the nucleus.

Interestingly, the participation of p19 in the aberrant growth properties of the E24 cells is questionable, when considering the shape of these cells in comparison to fibroblasts which have been transformed separately by E1A+E1B, E1A+p19, and E1A+p55 combinations (White and Cipriani, 1990) (figure 22). This comparison revealed a morphology which is specific to transformation by the E1A+p55 combination, suggesting the absence of p19. The selective expression of p55 could be explained by failure of the translational machinery to recognize the p19 AUG codon in the respective reading frame.

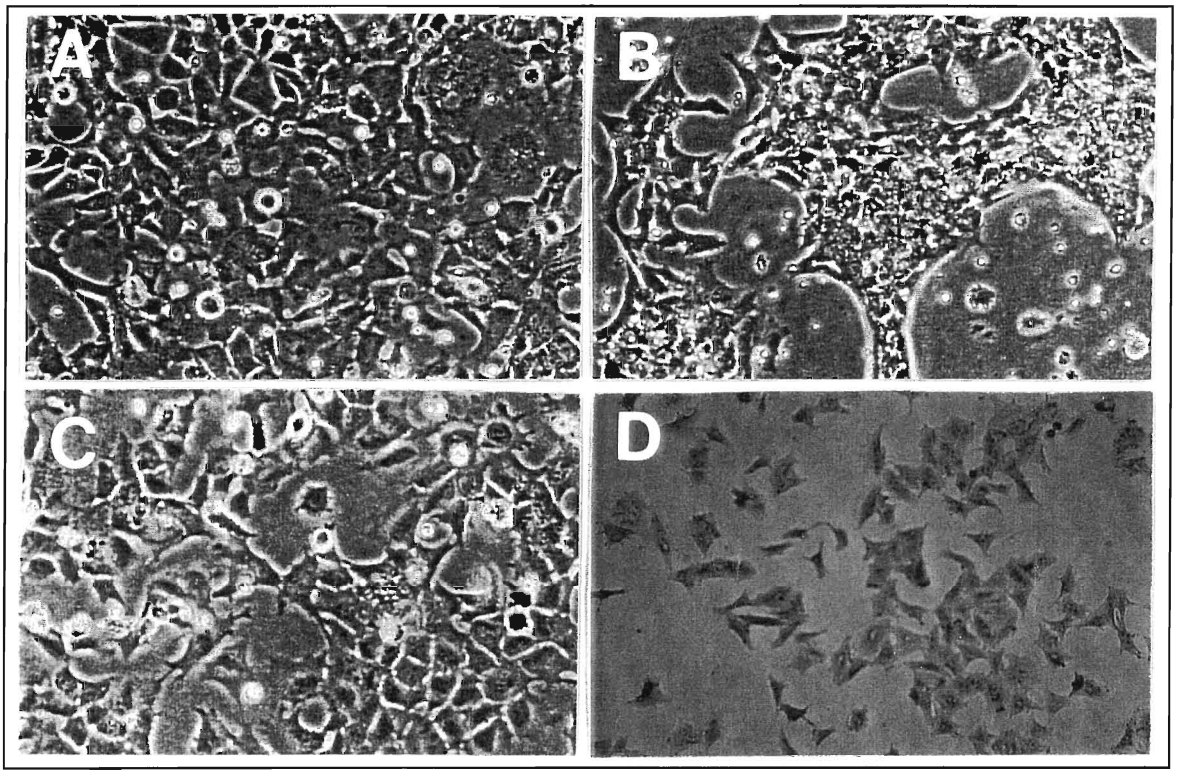


Figure 22. Comparison of E24 cells (D) with baby rat kidney fibroblasts which have been transformed by combinations of (A) E1A+E1B, (B) E1A+p19 and (C) E1A+p55 (White and Cipriani, 1990).

Since the effects of p19 and p55 are additive (White and Cipriani, 1990), the absence of either of these products could explain the inability of the E24 cells to grow to high density and beyond a monolayer, unlike fully transformed cells. In addition, cells which have been transformed by E1A+p55 have a flattened, trapezoidal shape, similar to E24 cells, whereas those transformed by E1A+p19 have a spindle shape (figure 22(B,C)). Those cells transformed by E1A and the complete E1B, on the other hand, exhibit combinations of both phenotypes (figure 22(A)), a morphology which was not apparent in the E24 cells (figure 22(D)).

In the absence of p19, the non-adherent nature of the E24 cells would have to be explained by a mechanism secondary to expression of the remaining E1 products. Phenotypic changes resulting from phosphorylation cascades or the activation of cellular oncogenes, is characteristic of transformed cells, where phosphorylation of tyrosine residues on the carboxy terminal of integrins can result in the release of the integrin from the cytoskeletal actin filaments, causing a subsequent release from the fibronectin and collagen matrix on the surface of the culture dish (Lui and Pawson, 1994). Incidentally, the rounding of cells due to phosphorylation of the integrins is also observed during mitosis, in which serine residues are the targets.

Similarly, transformed cells generally produce less fibronectin (Hynes, 1973), which normally links the extracellular collagen and the integrins, resulting in recruitment of actin filament bundles (stress fibers) to the focal adhesion contacts. Reduced production of

fibronectin therefore would prevent the flattening of the cells (Yamada, 1991). If culture dishes are first coated with a fibronectin-rich substrate, or if the level of fibronectin builds up naturally over time, then such transformed cells will exhibit a greater frequency of attachment and focal adhesion.

Based on the assumption that the E24 cells were rounded due to transformation, yet still viable, an attempt was made to seed E24 cells from the medium of a culture dish containing these cells, two days after plating. Although not quantified in terms of efficiency, the seeding of the non-adherent cells was a successful demonstration of the ability of those cells to eventually attach to the substrate and undergo focal adhesion.

If expression of the E1 region is confirmed, then the E24 cell line may represent the first demonstration of transformation by the otherwise putative transforming region of BAV3 in culture.

An attempt to detect and confirm expression of E1 products was made using immunoprecipitation, using polyclonal antibodies raised to the carboxy terminus of the 12S mRNA product from Ad2, which should recognize the E1A products produced from the 13S, 12S, 11S and 10S transcripts as well, since all share a common carboxy terminus. The major 289R and 243R products of the 13S and 12S transcripts should have theoretical molecular weights of 32kDa and 26kDa, respectively. Immunoprecipitation of these products from Ad5-infected cells, however, produces bands ranging from 35-53kDa, with predominant bands at 45-48.5kDa and 50-52kDa (Rowe *et. al.*, 1983; Yee and Branton, 1985). The discrepancy in size is likely due to extensive post-translational modification, since

these proteins are highly phosphorylated *in vivo* (Gaynor *et. al.*, 1982; Yee *et. al.*, 1983; Yee and Branton, 1985). The result would be the recovery of multiple products, ranging from 35 to 53 kDa, from the *293* control cells.

The use of this assay to analyse the candidate cell lines was based on the assumption that the human Ad polyclonals would recognize the BAV products. Since cross-reactivity was not previously demonstrated, the assumption could only be based on the extent of homology between the Ad2 and BAV3 E1 nucleotide sequences (Zheng *et. al.*, 1994). The use of the Ad polyclonals was based primarily on their availability, and a demonstration of their cross-reactivity would have been not only informative in terms of the conservation of protein structure, but also would have been valuable for designing further investigations.

The option of first demonstrating cross-reactivity by western blot was of course an option, but immunoprecipitation should have produced the same results if performed with the proper antibody titre, as could be established using *293* cells. In addition, the possible low level of expression of this regulatory region in the BAV3-infected cell controls may require a more sensitive assay, such as immunoprecipitation.

A preferable alternative to immunoprecipitation would be an assay which would directly address the functionality of E1 products in the candidate cell lines. Reporter genes linked to the promoter for the E2 DNA-binding protein (DBP) would satisfy this objective, since the E1 products are necessary and strong activators of the E2 promoter. In addition, screening for any transforming functions of

the BAV sequences could be done by transferring the transfected cell cultures directly to soft agar, whereby transformed clones could be easily and readily separated from the cell population.

Regardless of whether or not the E1 region is being expressed in the E24 cell line, the apparent immortalizaiton of the primary lung cells will permit the exploitation of their ease of transfection. Considering that MDBK and E61 cells are rather refractive to transfection, an attempt to develop a cell line from primary cells was appropriate for the purpose of transfection efficiency alone. The substitution of MDBK cells, the parent of E61 and a widely used bovine cell line, is desirable, given the low level of DNA uptake and recombination observed in these cells. The E24 cells may very well prove to be a rather useful research tool.

The different efficiencies of transfection among the various cell types used in this investigation could possibly be explained by membrane lipid profiles (particularly in the application of LIPOFECTAMINE™, a polycationic lipid) or perhaps even the intracellular environment. Explanations are only speculative, however, considering that the mechanism of DNA uptake for either method has not been completely elucidated. The case remains, nonetheless, that some cell types are not very amenable to DNA uptake.

Considering the ability of human Ads to transform cells in culture, an alternative to the methods used to generate the novel cell lines would be to infect a bovine culture with a human Ad, such as Ad5, or the highly oncogenic Ad12, whereby integration and immortalization would result from the non-permissive nature of the

infection. This approach would circumvent the need for subcloning the E1 region and for optimization of transfection conditions. The utility of the resulting cell line in the rescue of BAV vectors would rely on the ability of the human Ad products to complement growth of a BAV which, according to the results of the transactivation experiment of Zheng *et. al.* (1994) mentioned earlier, would be expected. One advantage of such a cell line would be the possible reduction of recombination between a mutant virus and the cellular genome, resulting in reversion to wild-type, as has been a problem with the human Ad and *293* cell line system.

Even if either the E61 or the E24 cells are expressing functional E1 products capable of complementing a BAV E1 deletion, it may still be necessary to develop additional cell lines, more efficient in terms of viral rescue, in light of the observation that overexpression of E1A products can inhibit viral replication (Adami and Babiss, 1990). It is therefore important to assess the level of E1 expression within the cells, and identify a candidate capable of efficient complementation and rescue. The inclusion of specific promoters to drive transcription of the E1 region may be a reasonable approach to addressing any problems involving levels of expression.

SUMMARY

Based on the results obtained in this experiment, the following conclusions can be stated:

(1) The left 0-19.7% of the BAV3 genome has been linked to the neomycin-resistance gene of *Tn5*, in a construct designated pKC-*neo*.

(2) The construct pKC-neo has been inserted into the genome of MDBK cells, generating the cell line E61, in such a way as to preserve the integrity of the BAV3 E1 sequences.

(3) The E61 cells seem to be transcribing the E1 region, as detected by Northern analysis of total RNA.

(4) The construct pKC-neo has also been inserted into the genome of primary bovine lung cells, generating the E24 cell line, but the orientation has not been confirmed.

(5) The E24 cells exhibit a morphology and growth property different from their parental cells and characteristic of transformed cells. They have lost the extended processes characteristic of primary fibroblasts, and they exhibit non-adherent properties.

(6) The E24 cells may represent the first demonstration of the transforming properities of the E1 region of BAV3 in culture.

(7) The E24 cells are efficient for the uptake of exogenous DNA, as compared to the MDBK cell line.

(8) The E24 cells may be a new cell line with potential for valuable applications to BAV research.

(9) Both the E61 and the E24 cell lines are candidates for the complementation and rescue of BAV mutants.

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